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(54) Title: POLYNUCLEOTIDE IMMOBILIZED SUPPORT

(57) Abstract

The present invention provides a nucleotide-immobilized support which includes an insoluble support and a polynucleotide bound to the support. This polynucleotide includes at least one sequence complementary to a polyadenylic acid tail of an mRNA. The nucleotide-immobilized support is useful for a variety of methods, including the synthesis of both sense and anti-sense cDNA, as well as double stranded cDNA. The nucleotide-immobilized support is also useful for synthesis of both sense and antisense mRNA. Additionally, the nucleotide-immobilized support also provides an improved method of placing a cDNA sequence into a vector in a particular orientation. The present invention also includes a new method for binding polynucleotides to insoluble supports.

POLYNUCLEOTIDE IMMOBILIZED SUPPORT

Background of the Invention

In most living organisms, genetic information is stored in the form of DNA. This DNA is transcribed into messenger RNA (mRNA) which then is translated into protein. In eukaryocytes, there is usually some loss of genetic information in the process of converting genomic DNA to the mature mRNA. This loss can be due to introns/exons, RNA splicing, or protein processing. Therefore, the genetic basis of protein structure is more advantageously studied using mRNA than genomic DNA.

Unfortunatley, mRNA is extremely unstable and easily digested by various ribonuclease (RNA digesting) enzymes (hereafter referred to collectively as "RNase") making experimentation difficult. Thus, many researchers have chosen to study DNA copies (cDNA) of interesting mRNA molecules. These copies are made using the enzyme reverse transcriptase (RT), isolated from retrovirus', which produces a single stranded DNA copy of the target mRNA.

Double stranded DNA (ds-cDNA) is generally more stable than single stranded cDNA (hereafter called "ss-cDNA"). The method of converting ss-DNA to ds-DNA using the enzyme DNA polymerase is well known in the art. The genetic information stored on ds-cDNA can then be used in various protocols, for example inserting ds-cDNA into expression vectors, which are then introduced into various cells. Promoters and restriction sites located on these vectors can then be used as tools for studying transcription and translation of the ds-cDNA.

In addition to using vector based promoters for transcription of mRNA nucleotides containing the polypeptide coding sequence (sense mRNA), antisense mRNA transcribed from the complementary cDNA strand can also be produced. Antisense mRNA is a useful tool for understanding biological function of a protein or mRNA whose function is unknown. Antisense RNA molecules are used to block production of specific proteins by anneling to the target mRNA and inhibiting translation. Therefore, it is anticipated that this type of translational inhibition will be important in the study of various gene products associated with a variety of diseases states.

Besides being a used for DNA polymerase based catalysis of ds-cDNA synthesis, ss-cDNA can be used as a template for polymerase chain reaction (hereafter called "PCR"). This method allows the rapid reproduction and enhancement of a specific gene sequence through the use of opposing nucleotide primers and a heat stable DNA polymerase. Multiple cycles of annealing and DNA synthesis rapidly produces many copies of the

targeted gene. Thus, sense ss-cDNA can be used to specifically amplify a segment of its corresponding ds-cDNA.

One currently known protocol for producing ds-cDNA is the liquid phase method described in Sambrook, et al., "Molecular Cloning, A Laboratory Manual, 2d Ed.," Cold
5   Spring Harbor Press, Cold Spring Harbor, NY (1989) (hereinafter referred to as "Molecular Cloning"). The complete disclosure of this manual is incorporated herein by reference thereto. In the liquid phase method, antisense ss-cDNA is produced from mRNA using reverse transcriptase, followed by mRNA digestion with RNase. Ds-cDNA is then synthesized from complementary chains of the remaining antisense ss-cDNA using DNA
10   polymerase. DNA polymerase reactions following reverse transcriptase don't require a specific primer since the RT leaves synthsized ss-cDNA with a self-priming loop structure at the 3' end.

With the liquid phase method, the resulting ds-cDNA does not contain a marker to identify the molecule's orientation. Accordingly, the newly synthesized ds-cDNA cannot be
15   readily inserted into a cloning vector since 50% of the clones will be in the wrong orientation for transcription. A rapid method of directionally cloning mRNA into a vector would therefore be desirable.

Ss-cDNA or ds-cDNA can also be produced using solid phase material. (I. Raineri et al. in *Nucleic Acids Research*, 19:4010 (1991)). In the solid phase method,
20   polydeoxythymidylic acid (poly dT), usually immobilized onto porous beads, is hybridized to the polyadenlic acid (poly A) tail of mRNA. Then, antisense ss-cDNA is synthesized from the bound polyadenelated mRNA by reverse transcriptase. After the template RNA is digested, second strand cDNA is synthesized by DNA polymerase. The resultant ds-cDNA has its antisense strand immobilized to the beads.

25   The solid phase method, however, yields product that cannot be disassociated from the insoluble support. In order circumvent this problem, the ss-cDNA can be released from the support-immobilized ds-cDNA by heating, and thereafter used to synthesize ds-cDNA using PCR. However, this adds another step to the reaction and requires an appropriate set of primers for every PCR reaction.

30   There is therefore a need for a simple method of creating unbound ds-cDNA clones from isolated mRNA. This method should preferably result in unbound, directionally cloneable products.

There are also various methods known of synthesizing mRNA from cDNA clones, one example is the liquid phase method which was described by S. Shichijo, et al. *in Journal*

of Neuroscience Research, 30:316-320 (1991). In this method, ds-cDNA is inserted into a vector containing a RNA promoter. The vector is then linearized by restriction enzyme digestion and mRNA is synthesized using RNA polymerase. The synthesized mRNA is then treated with a deoxyribonuclease (DNA digesting) enzyme (hereafter called "DNase") to remove the template DNA. If necessary, a polyadenelated tail can be added to the end of the newly synthesized RNA using the enzyme Terminal Transferase and dATP's.

The solid phase method of producing mRNA is also well known. Hironori Terada, et al., "Movement analysis of transcription by immobilized DNA," *Biophysics* 31:49-52 (1991) describe one such method. In this method, a DNA sequence is digested from the genome of bacteriophage λ by restriction enzyme cleavage, resulting in random DNA fragments with "sticky ends. T4 DNA polymerase is then used to fill in these sticky ends using biotinylated dUTP. The restriction enzyme is selected so as to leave a sticky end with an exposed dA nucleotide to which the biotinylated dUTP will hybridize during synthesis of DNA by the T4 DNA polymerase. Random sequences are then immobilized to an acrylamide support bearing avidin. mRNA can then be synthesized from those sequences bearing a natural λ promoter sequence using a polymerase T7 RNA polymerase or SP6 RNA polymerase. This system was designed to study the kinetic analysis of transcription in bacteriophage λ.

Both the liquid phase and solid phase methods of mRNA synthesis have disadvantages. The liquid phase method requires the use of a vector containing a RNA promoter, and also requires that the vector be converted into a linear sequence after insertion. The solid phase method does not always provide complete genetic information because the source of this information is immobilized genomic DNA and not mRNA. Thus, improved methods of mRNA synthesis would be desirable.

Stamm et al. in *Nucleic Acids Research*, 19:1350 (1991) described oligonucleotides covalently coupled via an amino link to a solid support for use in facilitating PCR experiments. However, the technique described by Stamm et al. does not specifically provide for the production of cDNA or RNA. Moreover, no mechanism is provided for removing the oligonucleotides covalenty coupled to the support.

<u>Summary of the Invention</u>

This invention is related to a polynucleotide immobilized support which is useful in the preservation and analysis of genes, the preservation method of genes using polynucleotide-immobilized support, and methods of synthesizing single stranded sense complementary DNA (hereafter called "cDNA"), double stranded cDNA (hereafter called "ds-cDNA"), sense messenger RNA ("mRNA") and of antisense mRNA.

One aspect of the present invention provides a nucleotide-immobilized support which includes an insoluble support and a polynucleotide bound to the support. This polynucleotide includes at least one sequence complementary to a polyadenylic acid tail of a mRNA. The nucleotide-immobilized support is useful for a variety of methods, including the synthesis of both sense and antisense cDNA, as well as double stranded cDNA. The nucleotide-immobilized support is also useful for synthesis of both sense and antisense mRNA. Additionally, the nucleotide-immobilized support provides an improved method of directionally placing a cDNA sequence into a vector. The present invention also includes new methods for binding polynucleotides to insoluble supports.

The invention focuses on a polynucleotide containing a sequence complementary to a polyadenylic acid tail of a mRNA, such as oligo (dT). In certain preferred aspects of the present invention, this polynucleotide can also contain recognition sites for restriction enzymes and RNA promoter sequences. The polynucleotide is generally immobilized on an Insoluble Dish such as microtiter plates. In one preferred form of the invention, the microtiter plate has at least two wells, each of which has a different polynucleotide immobilized therein. The polynucleotide-immobilized support of the present invention can be used to synthesize full length ds-cDNA, sense and antisense ss-cDNA, and sense and antisense mRNA. Furthermore, the immobilized ss-cDNA is stable for a long enough time to perform repeated rounds of ds-cDNA or mRNA synthesis.

One embodiment of the current invention is a nucleotide-immobilized support made of an Insoluble Dish (ID), which is preferably a microtiter plate, with a polynucleotide bound to the dish. The bound polynucleotide contains at least one sequence complementary to the polyadenylic acid tail of mRNA, for example poly(dt). In certain preferred embodiments, this polynucleotid also contains, at least one restriction enzyme site. In an even more preferred embodiment, this polynucleotide has an RNA promoter site in addition to the restriction enzyme site.   An alternative embodiment of the present invention is a nucleotide-immobilized support, preferably with a polynucleotide bound to an insoluble support containing at least one RNA promoter. The most preferable embodiment contains either the T7 or SP6 RNA promoter.

A further embodiment of the current invention is a nucleotide-immobilized support, made of an insoluble support, and a bound polynucleotide containing at least one restriction enzyme recognition site. Preferable restriction enzymes include EcoRI, Not I, Sma I and Sal I. A preferred sequence of the polynucleotide is one of SEQ ID NO:4, 13 or 14. In a

preferred embodiment. the bound polynucleotides are between 30 and 100 nucleotides in length.

In still another embodiment of the current invention, a ds-cDNA-immobilized support, made of ds-cDNA bound to an insoluble support, preferably an ID wherein at least one strand of the ds-cDNA contains at least one sequence complementary to the polyadenylic acid tail of mRNA. In a prefered embodiment of this invention, at least one strand of the ds-cDNA contains a RNA promoter, with a more preferable embodiment being wherein the ds-cDNA contains at least two different RNA promoters. More preferably, these promoters are either the T7 or SP6 RNA promoter. In an alternative embodiment of this ds-cDNA-immobilized support, at least one restriction enzyme recognition site is incorporated into the sequence.

Another separate embodiment of the current invention is a method for producing a ds-cDNA-immobilized support by binding a polynucleotide. preferably containing at least one RNA promoter, to an insoluble support. The polynucleotide preferably contains at least one sequence complementary to the polyadenylic acid tail of mRNA. thereby creating a nucleotide-immobilized support. Thereafter adding a solution. such as a cell lysate, containing polyadenylated mRNA to the nucleotide-immobilized support and permitting the polyadenylic acid tail of the mRNA to hybridize with the bound complementary sequence, and more preferably incubating the support and solution. thereby creating a first liquid phase and a solid phase. In a more preferable embodiment of the above method, the first liquid phase is removed after incubation. After hybridization. produce antisense cDNA complementary to the annealed mRNA. then produce sense cDNA complementary to previously produced antisense cDNA.

The highly prefered embodiment of the above method entails using reverse transcriptase. dATP. dCTP. dGTP and dTTP in the first reaction mixture, and then incubating the solid phase and first reaction mixture. After incubation. a second reaction mixture is added to the incubated solid phase containing RNase. DNA polymerase, DNA ligase, dATP. dCTP. dGTP and dTTP. Thereafter it is preferable to incubate the solid phase thereby resulting in a second liquid phase and then remove the second liquid phase.

One further embodiment of the present invention is drawn to a method for storing ds-cDNA which preserves the ds-cDNA by producing a ds-cDNA-immobilized support, removing any liquid from the support and thereafter storing the ds-cDNA-immobilized support.

Other alternative embodiments of the invention are of a method for producing antisense mRNA on a solid support, by producing a ds-cDNA-immobilized support as described above wherein the ds-cDNA bound to the support additionally contains a RNA promoter. Thereafter produce antisense mRNA by adding a sample solution containing
5   reagents which will allow the promoter to initiate transcription . The prefered method of producing antisense mRNA is, however, by incubating the support and sample solution, thereby creating a first liquid phase and a solid phase, and wherein the following steps are used to begin the reaction:

   1)   Add a reaction mixture to the solid phase containing RNA polymerase, ATP,
10   CTP, GTP and UTP with preferably one of the dNTP's being labeled.

   2)   Incubate the solid phase and reaction mixture then and

        heat the solid phase and reaction mixture, thereby   creating a liquid phase containing antisense mRNA.

Therafter it is prefered to continue the reaction by obtaining the liquid phase and treating
15   it with DNase.

Another embodiment of the present invention is drawn to a method for producing sense mRNA on a solid support by the following steps:

        (a)   binding a polynucleotide with at least one sequence complementary to the polyadenylic acid tail of mRNA, at least one RNA promoter, and at least one
20   restriction enzyme recognition site to an insoluble support, thereby creating a nucleotide-immobilized support;

        (b)   adding a sample solution containing mRNA with a polyadenylic acid tail to the nucleotide-immobilized support;

        (c)   permitting the polyadenylic acid tail of the mRNA to hybridize with
25   the complementary sequence;

        (d)   using the mRNA as a template to produce antisense cDNA complementary to the mRNA;

        (e)   producing sense cDNA complementary to the antisense cDNA, thereby creating a ds-cDNA molecule immobilized to the support;
30   (f)   adding a double stranded adaptor to the ds-cDNA, the adaptor containing at least one of each of the following: a second RNA promoter different from that on the polynucleotide bound to the insoluble support, and a second restriction enzyme recognition site different from the restriction enzyme recognition site on the polynucleotide bound to the insoluble support. Optionally, the reaction

mixture can be added to the solid phase containing DNA ligase and the double stranded adaptor;

(g)   digesting the ds-cDNA with a restriction enzyme which recognizes the second restriction enzyme recognition site;

(h)   producing a DNA/RNA duplex by transcribing RNA using the second RNA promoter. This reaction can be carried out by adding a mixture to the solid phase containing ATP, CTP, GTP and UTP, and an RNA polymerase which reacts with the second RNA promoter and thereafter incubating the solid phase and reaction mixture creating a DNA/RNA duplex. Optionally, one of the aforementioned dNTP's can be labeled; and

(i)   denaturing, preferably through heating, the DNA/RNA duplex, thereby creating a liquid phase containing sense mRNA and optionally treating the liquid phase with DNase.

Other Alternative embodiments of the present invention are drawn to methods of producing ds-cDNA by the follorwing steps:

1.   adding a sample solution containing mRNA to a nucleotide-immobilized support;

2.   incubating the sample solution and support, thereby creating a first liquid phase and a solid phase;

3.   removing the first liquid phase;

4.   adding a first reaction mixture containing reverse transcriptase, dATP, dCTP, dGTP, and dTTP to the solid phase, with one of the aforementioned nucleotides being labeled;

5.   incubating the first reaction mixture and solid phase;

6.   adding a second reaction mixture containing RNase, DNA polymerase, DNA ligase, dATP, dCTP, dGTP, and dTTP to the solid phase; and

7.   incubating the second reaction mixture and solid phase.

One other embodiment of the current invention is a method for producing sense ss-cDNA by creating ds-cDNA according to the method described above, then heating the second reaction mixture and solid phase. Thereby a second liquid phase will be created containing the obtainable sense ss-cDNA.

Other prefered embodiments of the current invention are to methods of producing antisense ss-cDNA by producing ds-cDNA as described above, then heating the second

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reaction mixture and solid phase. thereby creating a second liquid phase containing the sense ss-cDNA and thereafter obtaining the antisense ss-cDNA containing solid phase.

One other similar embodiment is a method for producing sense ss-cDNA by using the ds-cDNA-immobilized support described above. After the sense cDNA and antisense
5 cDNA have been allowd to form a ds-cDNA duplex in the liquid phase. the ds-cDNA is denatured. and the liquid phase is obtained.

Other similar embodiments of the current invention are drawn to a method for producing sense ss-cDNA. These methods include producing a ds-cDNA-immobilized support in a liquid phase, wherein one strand of the ds-cDNA is bound to an insoluble
10 support, and one strand of the ds-cDNA also contains at least one sequence complementary to the polyadenylic acid tail of mRNA. Thereafter. denature the ds-cDNA-immobilized support and obtain the liquid phase.

One further embodiment of the present invention is a method for producing antisense ss-cDNA. by producing a ds-cDNA-immobilized support as described above.
15 wherein the sense cDNA and antisense cDNA form a ds-cDNA duplex in the liquid phase. Preferably. the ds-cDNA contains a restriction enzyme site. which can optionally be cleaved in this method. After denaturing the ds-cDNA and removing the liquid phase. the resulting solid phase will contain the antisense ss-cDNA.

Other embodiments of this invention describe methods of manipulating cDNA
20 vectors by placing cDNA sequence into a vector in a particular orientation. One method is performed using the following steps:

(a)   producing a ds-cDNA-immobilized support as described above. wherein a polynucleotide bound to an insoluble support additionally contains a first restriction enzyme recognition site:

25 (b)   adding a double stranded adaptor to the ds-cDNA. the adaptor containing a second restriction enzyme recognition site different from the restriction enzyme recognition site on the polynucleotide bound to the insoluble support;

(c)   digesting the ds-cDNA with a restriction enzyme which recognizes the first restriction enzyme recognition site. and with a restriction enzyme which
30 recognizes the second restriction enzyme recognition site, thereby resulting in a non-bound ds-cDNA having a first sticky end at a first end thereof and preferably also a second sticky end at a second end thereof:

(d)   ligating the non-bound ds-cDNA to a vector having sequences complementary to the first sticky end at a first end thereof and preferably also sequences complementary to the second sticky end at a second end thereof.

An additional aspect of the current invention is drawn to a method of immobilizing a first single stranded polynucleotide having at least one purine base to an insoluble support having an exposed sulfhydryl residue by   hybridizing   the   first   single   stranded polynucleotide with a second single stranded polynucleotide in order to form a double stranded complex. The support is optionally treated with a primary amine compound. This complex will thereby protect the purine base from further reaction. Thereafter, react the 5' end of the first polynucleotide, preferably after denaturing the double stranded complex. with a maleimide compound to produce a polynucleotide having a maleimide group at its 5' end. One optional maleimide compounde is sulfo-SMCC. Further reactions will attach the maleimide group at the 5' end of the first polynucleotide with the sulfhydrl residue bound to the insoluble support.

In the method of attaching a polynucleotide to an insoluble support as described above. one prefered embodiment is wherein the sulfhydryl residue is produced from an exposed amine residue on the insoluble support by reacting the amine residue with SATA to form a reacted complex. Deacetylation of the reacted complex is then performed with hydroxylamine.

Brief Description of the Drawings

Figure 1 illustrates an immobilization procedure of a fist polynucleotide probe onto insoluble supports by the maleimide method.

Figure 2 illustrates an immobilization procedure of a first nucleotide probe onto insoluble supports by the carbodiimide method.

Figure 3 shows the overall principle of the procedure of synthesis of sense ss-cDNA, antisense mRNA and sense mRNA from the polynucleotide-immobilized support in this invention.

Figure 4 exhibits a Polaroid picture of agarose gel electrophoresis of the synthesized antisense mRNA (A) and sense mRNA (B) of Gs protein by the invented method.

Figure 5 exhibits a Polaroid picture of agarose gel electrophoresis of the PCR amplified ds-cDNA from ss-cDNA of Gs protein prepared from polynucleotide-immobilized support in this invention. (A). (B). and (C) indicate the first. second and third synthesis from the same plate.

Figure 6 exhibits a Polaroid picture of agarose gel electrophoresis of the PCR amplified ds-cDNA of jun oncogene from human peripheral blood leukocytes by this invented method.

Figure 7 exhibits a Polaroid picture of agarose gel electrophoresis of the PCR amplified ds-cDNA of various genes from human peripheral blood leukocytes by this invented method.   (A) and (B) indicate annealing temperature at 45°C and 55°C, respectively during PCR.

Figure 8 exhibits a Polaroid picture of agarose gel electrophoresis of the PCR amplified ds-cDNA of Gs protein by this invented method.

Detailed Description of the Invention

Definitions

The following well-known abbreviations are used herein:

A   : adenine

C   : cytosine

G   : guanine

T   : thymine

U   : uracil

dATP   : deoxyadenosine triphosphate

dCTP   : deoxycytidine triphosphate

dGTP   : deoxyguanosine triphosphate

dTTP   : deoxythymidine triphosphate.

For the purpose of this invention, an "Insoluble Dish" is defined as a concave or flat support device that is insoluble in the water-based buffers and solutions commonly used in the production of oligonucleotides.   Preferably, the Insoluble Dish used in the present invention is capable of holding liquids. Thus, a preferred embodiment includes a microtiter dish, a plastic plate or a nylon membrane, but not, for example a plastic or other insoluble bead.

Nucleotide sequences which can be specifically digested by restriction endonuclease enzymes are defined herein as "restriction enzyme recognition-sites." A large number of such recognition sites are known, and a given sequence may contain more than one recognition site. For example, some sequences might contain the recognition site for both NotI and for EcoRI.

An "RNA promoter" is defined herein as a nucleotide sequence which is recognized by RNA polymerase and can initiate transcription of nucleotides downstream therefrom. For example, the nucleotide sequence of the T7 promoter (Seq ID No. 1) is shown below:

5' - AATACGACTCACTATAG - 3'.

Another example of an RNA promoter is the SP 6 promoter (Seq ID No. 2), shown below:

5' - CATTTAGGTGACACTATAGAA - 3'.

An "insoluble support" is defined herein as a material which does not substantially dissolve in the solution used during a given procedure. and is not melted or dissolved during a heating procedure used in procedures within the scope of the present invention. Examples of insoluble supports would include an Insoluble Dish (including such supports as plastic microtiter plates, glass microtiter plates and nylon membranes). and would also include agarose and plastic beads.

In this invention, sense mRNA refers to the mRNA identical to the genetic information, while the antisense mRNA refers to a polynucleotide complementary to the sense mRNA. The sense ss-cDNA contains the identical genetic information to that of sense mRNA, although, in the cDNA the uracil bases in the mRNA are changed to thymine, and the nucleotides are deoxyribonucleotides. not ribonucleotides.

The water which is used in this invention is preferably treated with diethylpyrocarbonate (DEPC) in order to substantially reduce the activity of RNase therein. The DEPC treatment of water can be performed by adding 0.1% DEPC to water, incubating at 37°C overnight, followed by autoclaving.

Overview of the Present Invention

Using the present invention, ds-cDNA, ss-cDNA. sense mRNA. and antisense mRNA can be quickly and advantageously synthesized in an Insoluble Dish (ID) such as a microtiter plate from bound oligonucleotide sequences. The advantage of this system lies in the ease of changing solutions when the RNA is bound to an ID. Previously. ethanol precipitations or similar proceedures have been required with every solution change to recover the soluble RNA. In the preferred embodiment of the present invention, nucleotide sequences containing restriction enzyme sites. RNA promoter sequences and poly (dT) sequences are covalently bound to wells of a microtiter plate. Cell lysates containing cytoplasmic mRNA can be placed into the wells without further purification. In the preferred embodiment, an oligo (dT) nucleotide strand will hydrogen bond with the poly(A) tail of mRNA. While the

mRNA is immobilized by being annealed to its target, solution changes which do not interfere with the hybridization conditions can be performed.

Single-stranded-cDNA can be made from immobilized mRNA by adding deoxynucleotides and reverse transcriptase. Double-stranded-cDNA can then easily be produced by changing the medium to one containing DNA polymerase and deoxyoligonucleotides. Antisense mRNA can be transcribed by adding RNA polymerase targeted to the promoter binding site on the covalently linked oligonucleotide. Furthermore, adapters containing different restriction enzyme recognition sites and a second RNA promoter sequence can be preferably and advantageously ligated to the free end of the ds-cDNA.

Thereafter, sense mRNA can be transcribed quickly and easily by adding a RNA polymerase targeted to the ligated adapter. Single stranded sense cDNA can be repeatedly synthesized from immobilized single stranded antisense cDNA. This invention provides a useful tool for not only basic molecular biology and analysis. This invention can also be used for the molecular diagnosis and treatment in various diseases.

We have discovered that immobilization of polynucleotide sequences to an insoluble support containing at least one sequence complementary to a polyadenylic acid tail of mRNA can provide significant advantages in a variety of nucleotide synthesis and processing techniques useful in both theoretical and applied molecular biology. Many of these advantages will be made apparent from the ensuing description of the invention.

Bound Polynucleotides of the Present Invention

As discussed above, the immobilized oligonucleotides include nucleotide sequences complementary to the mRNA polyadenylic acid tail. Such complementary sequences would includ oligo (dT) as well as poly-U. It is preferable that the length of such sequences be between 15 and 80 bases, and more preferably between 30 and 100 bases.

Polynucleotide sequences of various lengths, including 30 to 100-mers, can be readily synthesized using techniques known to those of ordinary skill in the art. These techniques include methods for the synthesis of sequences having sequences complementary to the polyadenylic acid tail of mRNA, having an RNA promoter sequence, and/or having various restriction enzyme recognition sites. A DNA synthesizer can be used to expedite such synthesis. One such DNA synthesizer is manufactured by Applied Biosystems of Los Angeles, California.

In a preferred embodiment of the present invention, the polynucleotides including at least one sequence complementary to a polyadenylic acid tail of mRNA are bound to an

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insoluble support to create a nucleotide-immobilized support. Preferably, these sequences are from 30 to 100 nucleotides in length. In an especially preferred embodiment, the immoblized polynucleotides also include an RNA promoter, and/or restriction enzyme recognition sites. Preferably, the 5'-terminal end is bound to the insoluble support while the 3'-terminal end hybridizes to the polyadenylic acid tail of mRNA.

Thus, we have used a number of different polynucleotide sequences for immobilization. One particular sequence preferred sequence includes a poly (dT) sequence, Eco RI and Not I recognition sequences and the T7 promoter. The use of this sequence is described in many of the examples provided herein. However, we have also used other combinations of restriction enzyme recognition sites and promoters with similar results. For example, the use of Sma I-Sal I with the T7 promoter does not appear to significantly effect any of the methods described herein. We have also used Eco RI-Not I with the SP6 promoter.

Immobilization Methods

In the practice of the present invention, polynucleotides are immobilized onto an insoluble support. Various methods of immobilizing polynucleotides to the insoluble support are available, including covalent binding, ionic binding, and the physical absorbance method. However, the covalent binding method is preferred. Thus, in certain embodiments of the present invention, the polynucleotides are immobilized to microtiter plates which exhibit functional groups, such as carboxyl residues, amine residues, or hydroxyl residues on the surface thereof. Plastic plates exhibiting carboxyl residues or primary amine residues on the surface, or plastic plates which do not have these functional groups on the surface, but have them added later can also be used. Preferably, plastic plates which already express carboxyl residues or primary amine residues on the surface are used. Examples of plastic plates containing carboxyl residues and primary amine residues on the surface are the "Sumilon" microplates MS-3796F and MS-3696F, available from Sumitomo Bakelite.

In a preferred procedure for immobilization of the polynucleotide to an insoluble support exhibiting a functional group, the 5'-terminal end of the polynucleotide is covalently linked to the functional group. Any of a variety of methods for covalent binding of polynucleotides to these functional groups can be used. Examples of preferred well-known methods include the maleimide method and carbodiimide method.

The maleimide method, illustrated in Figure 1, involves the reaction between a substance containing a maleimide group and another material containing a sulfhydryl residue (SH). In order to attach the 5' end of a polynucleotide to an immobilized support using the

maleimide method, the 5' end of the polynucleotide is reacted with a maleimide compound. A suitable maleimide compound is sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC).

5   The SH residue is provided on the support by a reaction between a support having an amine residue and succinimidyl-S-acetylthioacetate (SATA), followed by deacetylation using hydroxylamine (NH$_2$OH). (Sulfo-SMCC and SATA are readily available from a variety of commercial sources, including the Pierce Company.) The resulting SH group on the support can then react with the maleimide group on the 5' end of the polynucleotide to form a polynucleotide-immobilized support.   One problem we have experienced in

10   the use of the maleimide method is that the SH group on the plate can react not only with an amine group at the 5' end of the polynucleotide, but can also react with primary amine groups on the purine bases, adenine and guanine. In order to assure that the polynucleotides are immobilized at their 5' ends, the amine groups on the purine bases can be protected by pairing the polynucleotide to a complementary polynucleotide prior to

15   immobilization. After immobilization, the complementary polynucleotide can be removed through denaturation, such as through heating.

The carbodiimide method is illustrated in Figure 2. This method involves a reaction between a carbodiimide compound with an amine residue and a material containing a carboxyl residue. An example of a carbodiimide compound is 1-ethyl-3-(3-dimethyl-

20   aminopropyl) carbodiimide hydrochloride (hereafter called EDC). In order to use EDC in the carbodiimide method, it must first be activated to an EDC compound containing an amine residue. This can be done by reaction with N-hydroxysulfosuccinimide (hereafter called Sulfo-NHS). Both EDC and Sulfo-NHS are available from well known commercial sources, including the Pierce Company.

25   In the practice of a preferred carbodiimide method for attaching polynucleotides to a support, a support having a carboxyl residue attached is used. EDC is activated by reaction with Sulfo-NHS. This activated EDC is reacted with support containing surface bound carboxyl residues. This can then be reacted with polynucleotides having an amine residue at their 5'-terminal ends, resulting in a polynucleotide-immobilized support.

30   We have found that non-specific binding of activated amine or carboxyl residues on insoluble supports can be effectively reduced or eliminated by treating plates to which polynucleotides have been immobilized with a primary amine compound, preferably glycine.

A large number of other immobilization methods can be used, including the biotin-avidin system described by Terada, et al., *supra*.

Application of Sample

In the preferred methods of the present invention, a sample containing mRNA is applied to the insoluble support to which a polynucleotide having a sequence complementary to a polyadenylic acid tail of mRNA has been bound. The polyadenylic acid tail of mRNA in the sample is allowed to hybridize with the bound polynucleotide. This can be accomplished by incubation at temperature dependent on a variety of factors, as is well known to those with ordinary skill in the art. These factors include the length of complementary nucleotide sequences, the ratio of Guanine or Cytosine (GC contents) within the entire base content in the complementary nucleotide sequences (which will be 0% if only the polyadenylic acid tail is complementary), the NaCl concentration in buffer, the number of bases which mismatch in the complementary nucleotide sequence, and the type of nucleotide. In a preferred form of this invention, the following equation can be used to calculate the preferred incubation temperature:

$$T_{inc} = 16.6 \times \log (M) + 0.41 \ (GC) + 81.5 - 67.5/n - 15 \ (°C).$$

In the equation shown above, M is the NaCl concentration (M) in solution, GC represents GC contents (%), n represents the length of nucleotide sequences (the number of nucleotides).

The incubation time can also be determined according to methods described in the Molecular Cloning manual.

The time for incubation is preferably from 1 hour to overnight, and the sample should preferably be gently swung during incubation. Incubation is preferably performed in an appropriate buffer solution. The same buffer used to hybridize RNA and DNA in the Northern Blot or the Dot Blot Methods can be used. The buffer is preferably prepared in a way so as not to contaminate it with RNase. If any RNase contamination is present, the activation thereof should be controlled to be as low as possible. RNase-free buffers are commercially available, such as those available within mRNA purification kits, such as those sold under the trademark "FastTrack" by InVitrogen of San Diego, California, or lysis buffer, which is described hereinbelow.

As stated above, in order to eliminate RNase activity from water used in the methods of this invention, the water is preferably treated with Diethylpyrocarbonate (DEPC). The preferred DEPC treatment involves addition of 0.1% DEPC to the water, followed by storage overnight at 37°C and sterilization in an autoclave.

Washing Procedures

Preferably, after incubation, unbound components of the sample are washed from the insoluble support. Appropriate solutions for washing include the incubation buffer or the buffer included in the mRNA purification kit. However, it is preferable to use a proper solution depending on the type of nucleic acid. In order to retain mRNA, the washing solution is preferably DEPC-treated 20 mM Tris, pH 7.5 containing 1 mM EDTA and 0.5 M of NaCl (hereafter called RNA washing solution). To retain DNA, the washing solution can be autoclaved 20 mM Tris, pH 7.5 containing 1 mM EDTA and 0.5 M of NaCl (hereafter called DNA washing solution).

Double-Stranded-cDNA Immobilized Support

The ds-cDNA immobilized support in this invention can be obtained as shown below. The polynucleotide-immobilized support is mixed with a mRNA sample allowing hybridization between the bound polynucleotide and the mRNA to occur. cDNA is then synthesized from the mRNA template using reverse transcriptase. The mRNA is preferably digested with RNase H, and ds-cDNA is then synthesized, preferably by DNA polymerase, the four dXTP's and ligase. As a result, a construct of ds-cDNA immobilized to the insoluble support is formed.

The complementary strand of DNA produced can be labeled by incorporating labeled dXTP's during the production thereof. Any of a variety of labels can be used, for example, radionuclides or agents, such as biotin, which can be detected by subsequent colorimetric or light-emitting reactions.

The bound cDNA can be used for a variety of further techniques. In accordance with a particular preferred method of the present invention, antisense RNA can be produced if the bound polynucleotide contains an RNA promoter sequence. An appropriate RNA polymerase for the promoter is addedd along with the four ribonucleotide triphosphates. As will be apparent to those with ordinary skill in the art, this antisense RNA can be labeled if at least one of these nucleotide triphosphates bears a label.

In accordance with an additional preferred method of the present invention, an analysis equivalent to RNA blot analysis can be performed by allowing a sample to hybridize with the bound antisense ss-cDNA resulting after heat denaturation. After heat denaturation, the sense ss-cDNA will be in the liquid phase. This sense ss-cDNA can be used for a variety of well-known techniques, such as PCR and sequencing.

The mRNA used in these experiments are preferably purified RNA, mRNA from biological samples, or RNase inhibited cell lysates. The purification of mRNA follows the

1 6

current mRNA purification protocol used for Northern blot or dot blot methods. Commercial kits are also available. For procedures involving RNase inhibited cell lysates, a lysis buffer is preferably used in which the biological material is treated with a pH 8.0 solution containing 200 μg/ml of Proteinase K and 10 mM of Vanadyl ribonucleoside

5   complex, 500 units/ml of RNase inhibitor, 0.5% of sodium dodecyl sulfate (SDS), and ethylene diamine tetra acetate (EDTA).

The incubation temperature vaires in each case depending on whether the experiment is a hybridization of nucleic acids or enzymatic reaction. Hybridization is performed as described above in the section entitled Application of Sample.

10   In the case of an enzymatic reaction, any temperature in which the enzyme is not mostly inhibited is acceptable, however, preferably the optimum temperature for each enzyme is used. The incubation time can be from 30 minutes to overnight either with or without shaking. In enzymatic reactions, any buffer which will not mostly inhibit enzyme activity is acceptable, however, the optimum buffer components for each enzyme activity are

15   most preferable. It is important however not to contaminate any of the buffers with RNase. If RNase is present, its activities should be controlled with inhibitors such as RNAsin to be as minimal as possible.

In addition, heating reactions, such as for denaturation of double-stranded polynucleotide complexes, are done in appropriate buffer solutions.

20   The insoluble support containing immobilized ds-cDNA can be stored for at least one month at 4°C under conditions where the solution is not contaminated with DNase and consists of preferably 20 mM Tris-hydrochloride, pH 7.6 containing 1 mM EDTA and 0.5 M NaCl.

The insoluble supports having immobilized polynucleotide can be reused many times.

25   In fact, we have reused the nucleotide-immobilized supports many times without significant loss of bound nucleotide. Digestion and removal of hybridized mRNA from the cDNA for reuse of the bound polynucleotide can be conducted by rinsing with a RNase containing solution after incubation. A mild NaOH solution in place of the RNase containing solution can also be used to remove hybridized mRNA from bound cDNA.

30   **Second RNA Promoter and Sense RNA Synthesis**

In certain embodiments of the methods of the present this invention, a second RNA is promoter is desired. This second RNA promoter can be used to direct the production of sense RNA. Use of labeled nucleotide triphosphates can be used to produce labeled sense RNA. The nucleotide sequence of the second RNA promoter is a sequence

recognized by RNA polymerase that can be used to initiate transcription of the subsequent downstream genetic information. The second RNA promoter is generally located on the opposite side of the ds-cDNA from the first promoter. These sequences preferably encode the binding site of a different promoter than the first promoter sequence in the polynucleotide-immobilized insoluble support. In this invention, the RNA polymerase which reacts with second RNA promoter should not cross react with the first RNA promoter sequence. If the first RNA promoter is for example, from the bacteriophage T7, the second promoter can be, for example, the SP6 promoter.

In order to provide a second promoter, an adaptor can be used which contains both the second RNA promoter and a second restriction enzyme recognition site. This adaptor can then be added to the end of the ds-cDNA. An example of an appropriate adaptor is the SalI-SP6 adaptor, as shown below (Seq ID No. 3):

```
5'-TCGACATTTAGGTGACACTATAGAA-3'
3'-    GTAAATCCACTGTGATATCTT-5'
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The second restriction enzyme recognition site should preferably not be the same sequences as those in the polynucleotide-immobilized insoluble support or the directionality of the digested mRNA will be lost. If the first restriction enzymes are, for example EcoRI and/or NotI, the second restriction enzymes could be SmaI and/or SalI.

Directional Cloning

A ds-cDNA molecule containing a first restriction enzyme site at one end and second restriction enzyme site at the other can be prepared by blunt end ligation. In this method, double stranded oligonucleotides containing a restriction enzyme site and RNA promoter sequence are ligated to the unbound end of the ds-cDNA using an enzyme such as T4 ligase. The resultant modified ds-cDNA can be inserted into a linearized vector containing both restriction enzyme sites. Because the first and second restriction enzyme sites are disimilar, the ds-cDNA ligation will be unidirectional. In this way the direction of cDNA insertion into the vector can be controlled.

Schematic Examplary Uses of the Invention

A schematic representation of a preferred embodiment of the present invention is shown in Figure 3. The insoluble immobilized support (a) containing a 30-100-mer polynucleotide sequence having a restriction enzyme recognition site, an RNA promoter and a oligo (dT) sequence is mixed with the sample solution containing polyadenelated mRNA. The resultant immobilized polynucleotide-mRNA complex is reacted with reverse transcriptase using the oligo (dT) tail of the bound nucleotides as a primer and the mRNA .

as a template. As a result, a mRNA-cDNA hybrid is synthesized on the insoluble support (c). RNase and DNA polymerase addition causes digestion of the RNA and conversion of the mRNA-cDNA hybrid into ds-cDNA affixed to the insoluble support (d). This immobilized ds-cDNA is then incubated at a low salt concentration in, for example, water, and then heated to denature the ds-cDNA. The sense ss-cDNA will be unable to maintain hydrogen bonding with its homologous pair and float free in solution (e). The unbound sense ss-cDNA (e) can thereafter be used as a PCR template to amplify specific genes of interest.

An alternative use of the aforementioned ds-cDNA immobilized support (d) is to produce antisense mRNA (f). RNA polymerase specific for the antisense strand of the ds-cDNA is used to transcribe RNA molecules . Antisense mRNA is then isolated by heating the microtiter plate, removing the liquid phase, and digesting with RNase-free DNase (f).

A further embodiment of the present invention uses the ds-cDNA immobilized support (d) as a ligation target for double stranded oligonucleotides. These oligonucleotides preferably contain nucleotide sequences corresponding to a second RNA promoter and second restriction enzyme site (g). This complex (g) can then be used to synthesize sense mRNA by incubating with the RNA polymerase which transcribes from the second promoter. Sense mRNA is then isolated by heating the microtiter plate, removing the liquid phase, and digesting the liquid phase with RNase-free DNase (h).

Various aspects of the invention are explained in more detail with reference to the following examples. These examples are intended solely to illustrate the invention, and not to limit the invention in any manner.

Example 1

Synthesis of ss-cDNA, ds-cDNA, sense and antisense mRNA, and PCR amplification of Gs protein-specific ds-cDNA from *in vitro* synthesized Gs protein-specific mRNA.

(1)   Synthesis of polydeoxyribonucleotide. A 53-mer polydeoxyribonucleotide containing two different restriction enzyme sites, EcoRI and NotI, the T7 RNA promoter sequence, and a series of 17 thymine residues from the 5' to 3' end were synthesized using a DNA synthesizer (Model 380B, Applied Biosystems). The sequence of this polydeoxyribonucleotide (Seq ID No. 4) is as follows:

5'-AGCTGAATTC GCGGCCGCAA TACGACTCAC TATAGTTTTT TTTTTTTTTT TTT-3'

According to the Applied Biosystems' protocol, a primary amine residue was also introduced at 5' end of this polydeoxyribonucleotide by using "Aminolink-2". (Applied

Biosystems). After synthesis. the polydeoxyribonucleotide was treated with approximately 30% ammonium hydroxide at 55°C overnight. dried by a Speed-vac vacuum centrifuge (Savant). resuspended in DEPC-treated water at 1 mg/ml. then stored at -20°C.

(2) Immobilization of polydeoxyribonucleotide onto the microtiter plate. 24 μl each of 20 mM EDC (Pierce) and 10 mM sulfo-NHS (Pierce) prepared in DEPC-water were mixed with 2 μl (2 μg) of the synthesized polydeoxyribonucleotide from step (1). and added into a microtiter plate well (MS-3796F. Sumitomo-bakelite). After incubation at 37°C overnight, the reaction solution was replaced with 20 mM Tris. pH 7.6 containing 1 mM EDTA and 0.5 M NaCl and stored at 4°C.

(3) Preparation of mRNA. Rat mRNA for the Gs protein was prepared as follows:

100 μg of the plasmid vector pGEM2 containing rat Gs protein cDNA was provided by Dr. R.R. Reed (John Hopkins University). The plasmid was linearized with 1000 units of Nhe (Promega) at 37°C for 2 hours. After extraction with an equal volume of a mixture containing Tris. pH 7.4-saturated phenol. chloroform. isoamylalcohol at a ratio of 25:24:1. the DNA was precipitated on dry ice for 30 min by addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ice-cold ethanol. The DNA was centrifuged at 15.000 rpm for 20 min at 4°C (MTX-150. Tomy).

The pellet was resuspended in 1 ml of 70% ethanol and recentrifuged at 15,000 rpm for 5 min at 4°C. After removal of the supernatant. the pellet was dried in a Speed-vac vacuum centrifuge (Savant) and resuspended in DEPC-water. Into the same tube, 20 μl of 5x RNA transcription buffer (Promega). 10 μl of 100 mM DTT. 100 units of RNase inhibitor (RNasin, Promega), 20 μl of 2.5 mM ribonucleotide mixture (Promega). and 100 units of T7 RNA polymerase (Promega) was added. The final volume was adjusted to 100 μl by addition of DEPC water. After incubatation at 37°C for 2 hours. 100 units (100 μl) of RNase-free DNase (Promega) was added and the reaction was continued at 37°C for an additional 30 minutes.

After DNA digestion. the reaction mixture was then extracted once with an equal volume of a mixture containing water-saturated phenol. chloroform. and isoamylalcohol at a ratio of 25:24:1. The synthesized RNA was precipitated on dry ice for 30 min by adding 0.1 volume of 3 M sodium acetate and 2.5 volume of ice-cold ethanol. The RNA was then centrifuged at 15.000 rpm for 20 min at 4°C (MTX-150. Tomy). and the pellet was resuspended in 1 ml of 70% ethanol and recentrifuged at 15.000 rpm for 5 min at 4°C. After removal of the supernatant. the pellet was dried in a Speed-vac vacuum centrifuge

(Savant). and resuspended in DEPC-water. The optical density of this RNA was determined by spectrophotometry at a wavelength of 260 nm (U-2000, Hitachi).

The RNA concentration was adjusted at 1 mg/ml based upon the calculation of 1.0 $OD_{260} = 40$ μg/ml. Five μl aliquots were then stored at -70°C until use.

5   (4).   ds-cDNA synthesis on microtiter plates. The synthesized Gs mRNA was diluted with DEPC-water, and 5 μl samples containing 5 μg, 1 μg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg or 0.1 pg of Gs mRNA was mixed with 45 μl of lysis buffer and incubated at 45°C for 1 hour to inactivate any remaining RNase activity. 5 M NaCl was added into each tube to make a final NaCl concentration of 0.5 M. Solutions were then placed into the

10   polydeoxyribonucleotide-immobilized microtiter wells. After incubation at room temperature for 30 minutes to allow hybridization of the bound ologo(dT) polydeoxyribonucleotide to the poly(A) tail of the mRNA, each well was washed twice with RNA washing buffer.

After washing, 50 μl of 50 mM Tris-HCl, pH 8.3 containing 75 mM KCl, 3 mM

15       $MgCl_2$, 10 mM DTT, 0.5 mM each of dATP, dCTP, dGTP and dTTP (in RT buffer) and 500 units of reverse transcriptase (Superscript, Gibco-BRL) was then added into each well to begin synthesis of the first cDNA strand. This reaction was incubated at 37°C for 1 hour.

After this "first strand" cDNA synthesis, the buffer was replaced with 50 μl of 25 mM

20   Tris-HCl, pH 7.5 containing 100 mM KCl, 5 mM $MgCl_2$, 10 mM ammonium sulfate, 0.15 mM β-nicotinic amidoadeninedinucleotide + (β-NAD+), 0.25 mM each of dATP, dCTP, dGTP and dTTP, 1.2 mM DTT, 65 units/ml DNA ligase and 250 units/ml DNA polymerase, 13 units/ml RNase H (Gibco-BRL) (dc-cDNA synthesis buffer), and incubated at 16°C for 3 hours to synthesize ds-cDNA from the first strand synthesis previously

25   performed. After incubation, the solution was replaced with 20 mM Tris-HCl, pH 7.6 containing 1 mM EDTA and 0.5 M NaCl, and stored at 4°C.

(5)   Preparation of ss-cDNA from bound nucleotides. The bound ds-cDNA synthesized as described in Paragraph (4) was washed with cDNA washing buffer 5 times, then incubated in 50 μl of DEPC-water at 80°C for 10 minutes to heat denature the

30   complementary cDNA strands. Solutions containing the unbound ss-DNA were immediately transferred to fresh 0.65-ml tubes, and stored at -20°C. Plates were reused for synthesizing ds-cDNA by again incubating with ds-cDNA synthesis buffer, as described in Paragraph (4) at 16°C for 3 hours. This procedure, repeated three times, prepared three ss-cDNAs from a single well.

2 1

(6)   Antisense mRNA synthesis. The ds-cDNA synthesized as described in Paragraph (4) was washed with RNA washing buffer 5 times, and incubated in 10 μl of 5x RNA transcription buffer (Promega), 5 μl of 100 mM DTT, 50 units (1.25 μl) of RNase inhibitor (RNasin, Promega), 10 μl of 10 mM ribonucleotide mixture (Promega), and 20 units (1 μl) of T7 RNA polymerase (Promega) to a final volume of 50 μl by addition of DEPC water.

After incubation at 37°C for 1 hour to allow transcription to begin, plates were heated to 90°C for 8 minutes, and the solutions containing unbound antisense mRNA were immediately transferred to fresh tubes. 10 units (10 μl) of RNase-free DNase (Promega) was added into the fresh tubes and the reaction continued at 37°C for 30 minutes to digest any remaining DNA.

The reaction mixture was then extracted once with an equal volume of a mixture containing water-saturated phenol, chloroform, and isoamylalcohol at a ratio of 25:24:1. Synthesized RNA was ethanol precipitated and centrifuged at 15,000 rpm for 20 minutes at 4°C (MTX-150, Tomy). The pellet was resuspended in 1 ml of 70% ethanol, and recentrifuged at 15,000 rpm for 5 minutes at 4°C. After removal of the supernatant the pellet was dried in a Speed-vac vacuum centrifuge (Savant), and resuspended in 5 μl DEPC-water.   The resultant antisense mRNA molecules were analyzed by agarose gel electrophoresis as follows:

Five μl of mRNA was mixed with 2 μl of 0.1 M MOPS pH 7.0 containing 40 mM sodium acetate, 5 mM EDTA, pH 8.0 (5X formaldehyde gel buffer), 10 μl formamide, 3.5 μl formaldehyde, and heated to 45°C for 15 minutes The mRNA solutions were then mixed with 2 μl of 50% glycerol containing 10 mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF (formaldehyde gel loading buffer), 1 μl of 1 mg/ml ethidium bromide, and loaded onto a 1% agarose gel. Electrophoresis was carried out in 10 mM phosphate buffer, pH 7.0 at 100 V/6.5 cm width for approximately 1 hour.   After electrophoresis, gels were exposed to UV light and flourescent RNA bands were recorded on Polaroid film as shown in Figure 4 (A).

In Figure 4 (A), lane 1 indicates the control Gs protein mRNA as described in Paragraph (3). Lanes 2 and 3 indicate two separate experiments of synthesized mRNA from this paragraph (6). The size of the synthesized mRNA was approximately 2000 bases, slightly larger than the control mRNA, probably due to polydeoxyribonucleotide addition at its 3' end.

2 2

(7)   <u>Sense mRNA synthesis</u>. Microtiter wells with bound ds-cDNA as described in paragraph (4) were washed with cDNA washing buffer 3 times, incubated in 10 µl of 5X ligase buffer (Gibco-BRL), 31 µl DEPC-water, 4 µl (4 µg) SalI-SP6 oligonucleotide adaptor (Seq ID No. 3) and 5 units (5 µl) of T4 ligase (Gibco-BRL) at 16°C overnight.

After washin each well three times with cDNA washing buffer, 5 µl of H buffer (Boerhinger-Mannheim), 44 µl of DEPC-water and 10 units (1 µl) of SalI (Boerhinger-Mannheim) were added. This solution was incubated at 37°C for 2 hours to allow cleavage at the SalI site. Each well was then washed with RNA washing buffer 5 times, and incubated in 10 µl of 5x RNA transcription buffer (Promega), 5 µl of 100 mM DTT, 50 units (1.25 µl) of RNase inhibitor (RNasin, Promega), 10 µl of 10 mM ribonucleotide mixture (Promega), and 20 units (1 µl) of SP6 RNA polymerase (Promega). The final volume was adjusted to 50 µl by addition of DEPC-water.

After incubating at 37°C for 1 hour, plates were heated to 90°C for 8 minutes, and the liquid solutions were immediately transferred to fresh tubes. 10 units (10 µl) of RNase-free DNase (Promega) was added and the reaction was continued at 37°C for an additional 30 minutes to digest any remaining DNA. The reaction mixture was then extracted once with an equal volume of a mixture containing water-saturated phenol, chloroform, and isoamylalcohol at a ratio of 25:24:1. Synthesized RNA was ethanol precipitated and the RNA was centrifuged at 15,000 rpm for 20 minutes at 4°C (MTX-150, Tomy). The pellet was resuspended in 1 ml of 70% ethanol and recentrifuged at 15,000 rpm for 5 minutes at 4°C. After removing the supernatant the pellet was dried and resuspended in 5 µl DEPC-water.

The resultant mRNA molecules were analyzed by agarose gel electrophoresis as described in paragraph (6), and flourescent RNA bands were recorded onto Polaroid films as shown in Figure 4 (B). Lanes 1, 2, and 3 of Figure 4 (B) indicate the control Gs protein mRNA as described in paragraph (3), and synthesized mRNA from paragraph (6) and this paragraph (7), respectively. The size of the synthesized mRNA was approximately 2000 bases, slightly larger than the control mRNA, probably due to addition of polydeoxyribonucleotides at the 3' and 5' ends of the molecule.

(8)   <u>Synthesis of PCR primers for amplification of G protein specific ds-cDNA</u>. Sense (G2-a) and antisense (G4-as) oligodeoxyribonucleotides specific for the G protein α subunit were synthesized in a DNA synthesizer as described in paragraph (1). The nucleotide sequences are as follows:

G2-s   5'-AGCACCATTGTGAAGCAGATGA-3' (Seq ID No. 5)

G4-as 5'-CTCTGGCCTCCCACATCAAACA-3' (Seq ID No. 6)

(9)   PCR amplification of cDNA. To 1 μl of ss-cDNA as described in paragraph

(5), add 0.1 μg (1 μl) each of oligonucleotide primers G2-s and G4-as. 5 μl of 10X PCR

buffer (Promega), 1 μl of 25 mM MgCl$_2$, 4 μl of 10 mM dNTP mixture (Promega), 0.5 μl

Taq polymerase (Promega), and 36.5 μl of DEPC-water. Two drops of mineral oil were

overlayered onto the reaction mixture to prevent evaporation. The reaction was first heated

to 95°C for 10 minutes, then PCR cycling was carried out in a Thermal cycler (Model 480,

Perkin-Elmer Cetus) with 30 cyles of annealing at 55°C for 1.5 minutes, extension at 72°C

for 4 minutes and denaturing at 95°C for 1.5 minutes

In a parallel experiment, Gs protein cDNA (10 ng in 1 μl) was used as a positive

control. Ten μl of the resultant PCR products were mixed with 1 μl of 10X loading buffer

(0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll type 400), heated to 65°C

for 5 minutes, then applied to a 1.5% agarose gel containing 5 μg/ml ethidium bromide.

Electrophoresis was carried out in a 1X TBE buffer (0.045 M Tris-borate buffer, pH 8.0

containing 0.001 M EDTA) at 100 V/6.5 cm width for approximately 1 hour. After

electrophoresis, nucleotide bands were fluoresced and recorded onto Polaroid film as shown

in Figure 5. In Figure 5, gels (A), (B), and (C) are the results from three consecutive

experiments. Lanes 2-10 of each gel contain 5 μg, 1 μg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg,

1 pg, and 0.1 pg of starting mRNA as described in paragraph (5). Lane 11 is a negative

control, and does not contain any mRNA. As shown in Figure 5, Gs protein DNA was

reproducibly amplified from starting mRNA concentrations of 10-100 pg with a size of

approximately 500 base pair, similar to that of the positive control in lane 1.

Example 2

Synthesis of ds-cDNA, sense ss-cDNA, and PCR amplification of the jun oncogene-

specific ds-cDNA from human leukocytes.

(I)   Polydeoxyribonucleotide-immobilized microtiter plate.

Polydeoxyribonucleotide-immobilized microtiter plates were prepared as described in

Example 1 paragraph (2).

(II)   Preparation of cell lysates from human leukocytes. Three mls of heparinized

blood were diluted three fold with phosphate buffered saline (PBS), and overlayered onto

3 mls IsoLymph (Gallard-Schlesinger) in a 15 ml tube. After centrifugation at 400 xg for

30 minutes, cells in the intermediate layer between IsoLymph and plasma were collected

into a fresh tube, and washed three times with PBS. 150 μl of lysis buffer was added to the

cellular pellet, and cells were completely lysed by repeated passage through an 18G needle.

Cell lysates were then incubated at 45°C for 30 minutes with intermittent shaking to reduce RNase activity.

(III)<u>Ds-cDNA synthesis on the microtiter plate</u>. A solution of 5 M NaCl was added to the cell lysate prepared in paragraph (II) and adjusted to a final concentration of 0.5 M. This solution was then diluted 10, 100 and 1000 fold with lysis buffer containing 0.5 M NaCl. Fifty μl each diluted sample was added into wells of the polydeoxyribonucleotide-immobilized microtiter plate, and incubated at room temperature for 30 minutes to allow hybridization between the bound poly-(dT) sequence and the poly(A) tail of the isolated cellular mRNA. After hybridization, the lysis buffer was removed, and each well was washed twice with RNA washing buffer. Fifty μl of RT buffer and 500 units of reverse transcriptase were added into each well and incubated at 37°C for 1 hour to initiate first strand cDNA synthesis. The buffer was then replaced with 50 μl of ds-cDNA synthesis buffer, and the plates were incubated at 16°C for 3 hours to cause ds-cDNA synthesis.

(IV)　<u>Preparation of ss-cDNA from the plates</u>. The ds-cDNA synthesized as described in paragraph (III) was washed with cDNA washing buffer 5 times, and incubated in 50 μl of DEPC-water at 80°C for 10 minutes to heat denature the compementary DNA strands. Liquid supernatents were then immediately transferred to fresh 0.65-ml tubes, and stored at -20°C.

(V)　<u>Synthesis of PCR primers for amplification of jun oncogene specific mRNA</u>. Sense (jun-s) and antisense (jun-as) oligodeoxyribonucleotides specific for jun oncogenes were synthesized in a DNA synthesizer as described in Example 1 paragraph (1). The nucleotide sequences were as follows:

jun-s　5'- CCC TGA AGG AGG AGC CGC AGA C -3'　　　　　(Seq ID No. 7)

jun-as　5'- CGT GGG TCA AGA CTT TCT GCT TGA GCT G -3' (Seq ID No. 8)

(VI)　<u>PCR amplification of cDNA</u>. To 1 μl of ss-cDNA as described in paragraph (IV), 0.1 μg (1 μl) each of jun-s and jun-as oligonucleotides. 5 μl of 10X PCR buffer (Promega), 1 μl of 25 mM $MgCl_2$. 4 μl of 10 mM dNTP mixture (Promega), 0.5 μl Taq polymerase (Promega), and 36.5 μl of DEPC-water were mixed. Two drops of mineral oil were overlayered onto the reaction mixture to prevent evaporation. The reaction mixture was first heated at 95°C for 10 minutes, then PCR was carried out in a Thermal cycler (Model 480, Perkin-Elmer Cetus) with 30 cycles of annealing at 55°C for 1.5 minutes,

extension at 72°C for 4 minutes and denaturing at 95°C for 1.5 minutes as described in Example 1 paragraph (9).

Ten μl of the resultant PCR products were then mixed with 1 μl of 10X loading buffer, and applied to a 1.5% agarose gel containing 5 μg/ml ethidium bromide. Electrophoresis was carried out in a 1X TBE buffer at 100 V/6.5 cm width for approximately 1 hour. After electrophoresis, fluorescent DNA bands were recorded onto Polaroid film as shown in Figure 4. Lanes 1-8 of Figure 6 indicate duplicate experiments of a 1:1000 dilution, full strength (equivalent to 1 ml of blood), 1:10, and 1:100 dilution respectively of the leukocyte lysate. As shown in Figure 6, the jun oncogene was amplified from the 1:10 and 1:100 dilution displaying an estimated size of 370 base pairs. This size is similar to the 372bp theoretical value.

Example 3

PCR amplification of the tachykinin receptor-specific ds-cDNA from human leukocytes.

i)   Synthesis of PCR primers for amplification of tachykinin receptors-specific mRNA. Sense (tac-s) and antisense (tac-as) oligodeoxyribonucleotides specific for the tachykinin receptor were synthesized using a DNA synthesizer as described in Example 1 paragraph (1). The nucleotide sequences were as follows:

tac-s   5'-GCCAGCATCTACTCCATGAC-3' (Seq ID No. 9)
tac-as 5'-GGGCAGCCACGAGATGG-3'   (Seq ID No. 10)

ii)   PCR amplification of cDNA. To 1 μl of ss-cDNA from cell lysates as described in Example 2 Paragraph (IV), 0.1 μg (1 μl) each of tac-s and tac-as oligonucleotides, 5 μl of 10X PCR buffer, 1 μl of 25 mM $MgCl_2$, 4 μl of 10 mM dNTP mixture, 0.5 μl Taq polymerase, and 36.5 μl of DEPC-water were mixed. Two drops of mineral oil were overlayered onto the reaction mixture to prevent evaporation. The reaction was first heated to 95°C for 10 minutes, then PCR was carried out in a Thermal cycler (Model 480, Perkin-Elmer Cetus) with 30 cycles of annealing at between 45°C and 55°C for 1.5 minutes, extension at 72°C for 4 minutes and denaturing at 95°C for 1.5 minutes as described in Example 1 paragraph (9). Ten μl of PCR products were then mixed with 1 μl of 10X loading buffer, heated to 65°C for 5 minutes, and applied to a 1.5% agarose gel containing 5 μg/ml ethidium bromide. Electrophoresis was carried out in a 1X

TBE buffer at 100 V/6.5 cm width for approximately 1 hour. After electrophoresis, the DNA bands were recorded onto Polaroid film. Results are discussed in Example 4 below.

Example 4

PCR amplification of the ornithine decarboxylase (ODC)-specific ds-cDNA from human leukocytes.

a) Synthesis of PCR primers for amplification of ODC-specific mRNA. Sense (OCD-s) and antisense (ODC-as) oligodeoxyribonucleotides specific for ODC were synthesized in a DNA synthesizer as described in Example 1 paragraph (1). The nucleotide sequences were as follows:

ODC-s   5'-GACTCTGGAGTGAGAATCATA-3' (Seq ID No. 11)
ODC-as   5'-ATCCAATCACCCACATGCATT-3' (Seq ID No. 12)

b) PCR amplification of cDNA. To 1 μl of ss-cDNA from cell lysates 0.1 μg (1 μl) each of ODC-s and ODC-as oligonucleotides, 5 μl of 10X PCR buffer, 1 μl of 25 mM MgCl$_2$, 4 μl of 10 mM dNTP mixture, 0.5 μl Taq polymerase, and 36.5 μl of DEPC-water were mixed. Two drops of mineral oil were overlayered onto the reaction mixture to prevent evaporation. The PCR reaction was carried out as described in Example 1 Paragraph (9). Ten μl of the PCR reaction products were then mixed with 1 μl of 10X loading buffer, heated at 65°C for 5 minutes, and applied to a 1.5% agarose gel containing 5 μg/ml ethidium bromide. Electrophoresis was carried out in a 1X TBE buffer at 100 V/6.5 cm width for approximately 1 hour. After electrophoresis, gels were exposed to UV light in order to fluoresce the DNA bands, and recorded onto Polaroid film as shown in Figure 7.

In Figure 7, lanes (A) and (B) indicate annealing temperatures of 45°C and 55°C. Lanes 1, 2 and 3 indicate primers for ODC, tachykinin receptors and jun oncogenes, respectively. As shown in Figure 7, the gene for ODC was amplified at both annealing temperatures of 45°C and 55°C while the genes for the tachykinin receptor and jun oncogenes were only amplified at 45°C.

Example 5

Construction of a cDNA library from ds-cDNA synthesized onto the polydeoxyribonucleotide-immobilized microtiter plates, and introduction of the cDNA library into bacteria.

(1)   Ligation of the SP6-SalI adaptor to ds-cDNA synthesized on the microtiter plate. Human leukocyte ds-cDNA synthesized on microtiter plates as described in Example 2 paragraph (III) were ligated with SP6-SalI adaptors, and the cDNA was then digested with SalI as described in Example 1 Paragraph (7).

(2)   Removal of ds-cDNA from the microtiter plate. After SalI digestion as described above, wells were washed with cDNA washing buffer 3 times, and incubated in 5 μl of H buffer, 44 μl of DEPC-water and 10 units (1 μl) of NotI (Boerhinger-Mannheim) at 37°C overnight to cleave the NotI site on the immobilized polydeoxyribonucleotide. The reaction mixture was then transferred to a fresh 0.65-ml tube, and heated at 65°C for 20 minutes to deactivate NotI.

(3)   Ligation of ds-cDNA into a vector (cDNA library construction). Ten μl of heat-denatured ds-cDNA as described above was mixed with 4 μl of 5X T4 ligase buffer, DEPC-water, 1 μl (50 ng) of NotI-SalI pre-digested pSPORT vector (Gibco-BRL), 1 μl of T4 ligase, and incubated at room temperature for 3 hours to ligate the cDNA and the vector. In order to precipitate the cDNA-vector complex, 5 μl (5 μg) of yeast tRNA (Gibco-BRL), 12.5 μl of 7.5 M ammonium acetate and 70 μl of ethanol was added into the reaction mixture and placed at -70°C overnight. The DNA was centrifuged at 15,000 rpm for 20 minutes at 4°C (MTX-150, Tomy), and the pellet was resuspended in 1 ml of 70% ethanol and recentrifuged at 15,000 rpm for 5 minutes at 4°C. After the supernatant was removed, the pellet was dried by a Speed-vac vacuum centrifuge (Savant), and resuspended in DEPC-water.

(4)   Transformation. A cDNA-ligated plasmid as described above was resuspended in 5 μl of DEPC water. One 1 μl was then mixed with 25 μl of competent cells (ElectroMax DH10a, Gibco-BRL) on ice and then placed into a 0.1 cm cuvette (BioRad). Electroporation was carried out at 16.6 kV/cm to incorporate plasmids into the cells. One ml of SOC medium was added into the cuvette, and cells were then transferred to a fresh 10-ml polypropylene tube. The cell mixture was incubated at 37°C for exactly 1 hour with constant shaking at 225 rpm. After cells were diluted 10 and 100 fold with SOC medium, 50 μl were spread onto Liquid Broth agar plates containing 100 μg/ml ampicillin and incubated at 37°C overnight. The number of colonies was then manually counted.

As shown in Table I, approximately 600,000-900,000 colonies were obtained from each well, representing 1 ml of blood leukocytes.

TABLE I

Experiment Number	Number of Colonies*
1	900,000
2	850,000
3	600,000

*: number of colonies from 1 ml of blood leukocytes.

This experiment shows that ds-cDNA can be amplified and subsequently cloned into a plasmid vector. This method is advantageously both efficient and fast.

Example 6

Synthesis of ss- and ds-cDNA, and PCR amplification of Gs protein-specific ds-cDNA from in vitro synthesized Gs protein-specific mRNA from different oligonucleotide-coupled plates.

(1)   Synthesis of polydeoxyribonucleotide. The 50-mer polydeoxyribonucleotide containing two different restriction enzyme sites such as SmaI and SalI, T7 RNA promoter sequence followed by a series of 17 thymine residues from 5' to 3' and were synthesized as described in Example 1. Actual sequence of this polydeoxyribonucleotide is as follows:

5'-GGGGCCCGGGGTCGACAATACGACTCACTATAGTTTTTTTTTTTTTTTTT-3' (SEQ ID NO:13)

The 56-mer polydeoxyribonucleotide containing two different restriction enzyme sites such as EcoRI and NotI, SP6 RNA promoter sequence followed by a series of 17 thymine residues from 5' to 3' end were synthesized as described in Example 1. Actual sequence of this polydeoxyribonucleotide is as follows:

5'-GGGGGAATTCGCGGCCGCCATTTAGGTGACACTATAGAATTTTTTTTTTTTT TTTT-3' (SEQ ID NO:14)

Primary amine residue was also introduced at 5' end of these polydeoxyribonucleotides by using Aminolink 2 as described in Example 1.

(2)   Immobilization of polydeoxyribonucleotide onto the microtiter plate. 24 μl each of 20 mM EDC (Pierce) and 10 mM sulfo-NHS (Pierce) prepared in DEPC-water

were mixed with 2 µl (2 µg) of the synthesized polydeoxyribonucleotide, and added into a well of the microtiter plate (MS-3796F, Sumitomo-bakelite). After the plate was incubated at 37°C overnight, reaction solution of each wells was replaced with 20 mM Tris, pH 7l.6 containing 1 mM EDTA and 0.5 M NaCl, and the plate was stored at 4°C until use.

5   (3)   <u>ds-cDNA synthesis on the microtiter plate.</u> The synthesized Gs mRNA as described in Example 1, was adjusted with lysis buffer containing 0.5 M NaCl at 2 µg/50 µl, and 50 µl of solution was added into the polydeoxyribonucleotide-immobilized microtiter well. After the plates were incubated at room temperature for 30 min. each wells were washed twice with RNA washing buffer. cDNA was then synthesized on the plate as

10   described in Example 1. using MMLV-reverse transcriptase instead of Superscript. After first strand cDNA synthesis, second strand cDNA was synthesized on the plate as described in Example 1. The ds-cDNA synthesized wells were washed with cDNA washing buffer 5 times, and incubated with 50 µl of DEPC-water at 80°C for 10 min for heat denature of DNA double helix. Solutions were immediately transferred to fresh 0.65-ml tubes, and

15   stored at 20°C until use.

        (4)   <u>PCR amplification of cDNA.</u> To 1 µl of ss-cDNA as described in (3), 0.1 µg (1 µl) each of G2-s and G4-as as described in Example 1, 5 µl of 10X PCR buffer, 1 µl of 25 mM MgCl, 4 µl of 10 mM dNTP mixture, and 0.5 µl Taq polymerase, and 36.5 µl of DEPC water were mixed, then 2 drops of mineral oil were overlayed onto the reaction

20   mixture to prevent evaporation. Tubes were first heated at 95°C for 10 min, then PCR was carried out in a Thermal cycler (Model 480, Perkin-Elmer Cetus) with 30 cycles of annealing at 55°C for 1.5 min as described in example 1. Ten µl of PCR products were then mixed with 1 µl of 10X loading buffer, heated at 65°C for 5 min. then applied to a 1.5% agarose gel containing 5 µg/ml ethidium bromide. Electrophoresis was carried out in a 1X TBE

● ●

buffer at 100 V/6.5 cm width for approximately 1 hour. After electrophoresis, gels were exposed to U.V. light to visualize DNA bands, and recorded onto Polaroid films as shown in Figure 6A. In Figure 6A, lanes 2 and 3 indicate the result of two consecutive experiments from plates immobilized with a polynucleotide having the sequence of SEQ ID NO:13, and

5   lanes 5 and 6 indicate the result of two consecutive experiments from plates immobilized with a polynucleotide having the sequence of SEQ ID NO:14. Lanes 1 and 4 are negative control from plates immobilized with a polynucleotide having the sequence of SEQ ID NO:13 and 14, respectively, without any applied mRNA.

Thus, the foregoing examples demonstrate the wide-ranging utility of the present

10   invention. However, as these examples are intended to illustrate, rather than to limit the present invention, the scope of the present invention is to be interpretted with reference to the appended claims.

SEQUENCE LISTING

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10

      (iii) NUMBER OF SEQUENCES: 12

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      (v) COMPUTER READABLE FORM:
          (A) MEDIUM TYPE: Floppy disk
          (B) COMPUTER: IBM PC compatible
          (C) OPERATING SYSTEM: PC-DOS/MS-DOS
25   (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

      (vi) CURRENT APPLICATION DATA:
          (A) APPLICATION NUMBER:
          (B) FILING DATE:
30   (C) CLASSIFICATION:

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35   (C) REFERENCE/DOCKET NUMBER: HITACHI.002A

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40

    (2) INFORMATION FOR SEQ ID NO:1:

      (i) SEQUENCE CHARACTERISTICS:
45   (A) LENGTH: 17 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: cDNA to mRNA

          (iii) HYPOTHETICAL: NO

5   (iv) ANTI-SENSE: NO

          (vi) ORIGINAL SOURCE:
              (A) ORGANISM: T7 Promoter Sequence

10

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

      AATACGACTC ACTATAG   17

15   **(2) INFORMATION FOR SEQ ID NO:2:**

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 21 base pairs
              (B) TYPE: nucleic acid
20   (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: cDNA to mRNA

25   (iii) HYPOTHETICAL: NO

          (iv) ANTI-SENSE: NO

          (vi) ORIGINAL SOURCE:
30   (A) ORGANISM: SP6 PROMOTER SEQUENCE

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

35   CATTTAGGTG ACACTATAGA A   21

     (2) INFORMATION FOR SEQ ID NO:3:

          (i) SEQUENCE CHARACTERISTICS:
40   (A) LENGTH: 25 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: double
              (D) TOPOLOGY: linear

45   (ii) MOLECULE TYPE: cDNA to mRNA

          (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
    (A) ORGANISM: SALI-SP6 ADAPTER SEQUENCE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCGACATTTA GGTGACACTA TAGAA   25

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 53 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA to mRNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: 53-MER OLIGO CONTAINING ECOR1, NOTI, AND T7
            PROMOTER

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCTGAATTC GCGGCCGCAA TACGACTCAC TATAGTTTTT TTTTTTTTTT
TTT   53

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA to mRNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
     (A) ORGANISM: G2-S OLIGONUCLEOTIDE PRIMER

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGCACCATTG TGAAGCAGAT GA   22

(2) INFORMATION FOR SEQ ID NO:6:
10
         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 22 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
15   (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: cDNA to mRNA

         (iii) HYPOTHETICAL: NO
20
         (iv) ANTI-SENSE: NO

         (vi) ORIGINAL SOURCE:
              (A) ORGANISM: G2-AS OLIGONUCLEOTIDE PRIMER
25

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCTGGCCTC CCACATCAAA CA   22
30
(2) INFORMATION FOR SEQ ID NO:7:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 22 base pairs
             (B) TYPE: nucleic acid
35   (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: cDNA to mRNA
40
         (iii) HYPOTHETICAL: NO

         (iv) ANTI-SENSE: NO

45   (vi) ORIGINAL SOURCE:
              (A) ORGANISM: JUN-S OLIGONUCLEOTIDE PRIMER

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCTGAAGGA GGAGCCGCAG AC   22

5

(2) INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 28 base pairs
10   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA to mRNA
15
        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

20   (vi) ORIGINAL SOURCE:
            (A) ORGANISM: JUN-AS OLIGONUCLEOTIDE PRIMER

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
25
CGTGGGTCAA GACTTTCTGC TTGAGCTG   28

(2) INFORMATION FOR SEQ ID NO:9:

30   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 20 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
35
        (ii) MOLECULE TYPE: cDNA to mRNA

        (iii) HYPOTHETICAL: NO

40   (iv) ANTI-SENSE: NO

        (vi) ORIGINAL SOURCE:
            (A) ORGANISM: TAC-S OLIGONUCLEOTIDE PRIMER

45
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCAGCATCT ACTCCATGAC   20

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(2) INFORMATION FOR SEQ ID NO:10:

　　(i) SEQUENCE CHARACTERISTICS:
　　　　(A) LENGTH: 17 base pairs
5　　　　(B) TYPE: nucleic acid
　　　　(C) STRANDEDNESS: single
　　　　(D) TOPOLOGY: linear

　　(ii) MOLECULE TYPE: cDNA to mRNA
10

　　(iii) HYPOTHETICAL: NO

　　(iv) ANTI-SENSE: NO

15　　(vi) ORIGINAL SOURCE:
　　　　(A) ORGANISM: TAC-AS OLIGONUCLEOTIDE PRIMER

　　(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
20
GGGCAGCCAC GAGATGG　　　　　　　　　　　　　　17

(2) INFORMATION FOR SEQ ID NO:11:

25　　(i) SEQUENCE CHARACTERISTICS:
　　　　(A) LENGTH: 21 base pairs
　　　　(B) TYPE: nucleic acid
　　　　(C) STRANDEDNESS: single
　　　　(D) TOPOLOGY: linear
30
　　(ii) MOLECULE TYPE: cDNA to mRNA

　　(iii) HYPOTHETICAL: NO

35　　(iv) ANTI-SENSE: NO

　　(vi) ORIGINAL SOURCE:
　　　　(A) ORGANISM: ODC-S OLIGONUCLEOTIDE PRIMER

40
　　(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACTCTGGAG TGAGAATCAT A　　　　　　　　　　21

45　(2) INFORMATION FOR SEQ ID NO:12:

　　(i) SEQUENCE CHARACTERISTICS:
　　　　(A) LENGTH: 21 base pairs

            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

5   (ii) MOLECULE TYPE: cDNA to mRNA

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO
10
        (vi) ORIGINAL SOURCE:
            (A) ORGANISM: ODC-AS OLIGONUCLEOTIDE PRIMER

15   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATCCAATCAC CCACATGCAT T   21

        (2) INFORMATION FOR SEQ ID NO:13:
20
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 50 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
25   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA to mRNA

        (iii) HYPOTHETICAL: NO
30
        (iv) ANTI-SENSE: NO

        (vi) ORIGINAL SOURCE:
            (A) ORGANISM: 50-MER OLIGO CONTAINING Sma I AND Sal I SITES AND
35   T7 RNA PROMOTER

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
40
GGGGCCCGGG GTCGACAATA CGACTCACTA TAGTTTTTTT TTTTTTTTTT   50

        (2) INFORMATION FOR SEQ ID NO:14:
45
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 56 base pairs
            (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

5

     (ii) MOLECULE TYPE: cDNA to mRNA

     (iii) HYPOTHETICAL: NO

     (iv) ANTI-SENSE: NO

10   (vi) ORIGINAL SOURCE:
        (A) ORGANISM: 56-MER OLIGO CONTAINING Eco RI AND Not I SITES
           AND SP6 RNA PROMOTER

15   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGGGAATTC GCGGCCGCCA TTTAGGTGAC ACTATAGAAT TTTTTTTTTT
TTTTT   56
20

WE CLAIM:

1.   A method for producing a ds-cDNA-immobilized support, comprising the steps of:

     (a)   binding a polynucleotide to an insoluble support, said polynucleotide comprising at least one sequence complementary to a polyadenylic acid tail of mRNA, thereby creating a nucleotide-immobilized support;

     (b)   adding a sample solution containing mRNA with a polyadenylic acid tail to said nucleotide-immobilized support;

     (c)   permitting the polyadenylic acid tail of said mRNA to hybridize with said sequence complementary to a polyadenylic acid tail;

     (d)   producing antisense cDNA complementary to said mRNA, using said mRNA as a template; and

     (e)   producing sense cDNA complementary to said antisense cDNA.

2.   The method of Claim 1, wherein said polynucleotide additionally comprises at least one RNA promoter.

3.   The method of Claim 1, wherein said sample solution comprises a cell lysate.

4.   The method of Claim 1, wherein step (c) comprises incubating said support and sample solution, thereby creating a first liquid phase and a solid phase.

5.   The method of Claim 4, additionally comprising removing the first liquid phase.

6.   The method of Claim 4, wherein step (d) comprises:

adding a first reaction mixture to the solid phase, said first reaction mixture comprising reverse transcriptase, dATP, dCTP, dGTP and dTTP; and

incubating said solid phase and first reaction mixture.

7.   The method of Claim 6, wherein step (e) comprises:

adding a second reaction mixture to the incubated solid phase, said second reaction mixture comprising RNase, DNA polymerase, DNA ligase, dATP, dCTP, dGTP and dTTP;

incubating said solid phase, thereby resulting in a second liquid phase; and

removing the second liquid phase.

8.   A method for storing ds-cDNA which preserves said ds-cDNA, comprising the steps of:

(i)   producing a ds-cDNA-immobilized support by the method of Claim

1;

(ii)   removing any liquid from said support; and

(iii)   storing said ds-cDNA-immobilized support.

5   9.   A method for producing antisense mRNA on a solid support, comprising the

steps of:

(i)   producing a ds-cDNA-immobilized support by the method of Claim

1, wherein the ds-cDNA bound to said support additionally comprises an RNA

promoter; and

10   (ii)   producing antisense mRNA from said promoter.

10.   The method of Claim 9, wherein step (c) comprises incubating said support

and sample solution, thereby creating a first liquid phase and a solid phase, and wherein

step (ii) comprises:

adding a reaction mixture to said solid phase, said reaction mixture

15   comprising RNA polymerase, ATP, CTP, GTP and UTP;

incubating said solid phase and reaction mixture; and

heating said solid phase and reaction mixture, thereby creating a liquid phase

containing antisense mRNA.

11.   The method of Claim 10, wherein at least some of the ATP, CTP, GTP or

20   UTP added in the adding step is labeled.

12.   The method of Claim 10, additionally comprising:

obtaining said liquid phase containing antisense mRNA ; and

treating said liquid phase containing antisense mRNA with DNase.

13.   A method for producing sense mRNA on a solid support, comprising the

25   steps of:

(a)   binding a polynucleotide to an insoluble support, said polynucleotide

comprising at least one sequence complementary to a polyadenylic acid tail of

mRNA, at least one RNA promoter and at least one restriction enzyme recognition

site, thereby creating a nucleotide-immobilized support;

30   (b)   adding a sample solution containing mRNA with a polyadenylic acid

tail to said nucleotide-immobilized support;

(c)   permitting the polyadenylic acid tail of said mRNA to hybridize with said sequence complementary to a polyadenylic acid tail;

(d)   producing antisense cDNA complementary to said mRNA, using said mRNA as a template;

5   (e)   producing sense cDNA complementary to said antisense cDNA, thereby producing a ds-cDNA immobilized to said support;

(f)   adding a double stranded adaptor to said ds-cDNA, said adaptor comprising at least one of each of the following: a second RNA promoter different from the RNA promoter on said polynucleotide bound to said insoluble support, and

10   a second restriction enzyme recognition site different from the restriction enzyme recognition site on said polynucleotide bound to said insoluble support;

(g)   digesting said ds-cDNA with a restriction enzyme which recognizes said second restriction enzyme recognition site;

(h)   producing a DNA/RNA duplex by producing RNA using said second

15   RNA promoter; and

(i)   denaturing said DNA/RNA duplex, thereby creating a liquid phase containing sense mRNA.

14.   The method of Claim 13, wherein step (f) comprises:

adding a reaction mixture to said solid phase, said reaction mixture

20   comprising DNA ligase and said double stranded adaptor; and

incubating said solid phase and reaction mixture.

15.   The method of Claim 13, wherein step (h) comprises:

adding a reaction mixture to said solid phase, said reaction mixture comprising ATP, CTP, GTP and UTP, and an RNA polymerase which reacts with

25   the second RNA promoter; and

incubating said solid phase and reaction mixture, thereby creating a DNA/RNA duplex.

16.   The method of Claim 15, wherein at least some of the ATP, CTP, GTP or UTP added in the adding step is labeled.

30   17.   The method of Claim 13, wherein step (i) comprises heating said DNA/RNA duplex.

18.   The method of Claim 13, additionally comprising:

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obtaining the liquid phase of step (i); and

treating said liquid phase with DNase.

19. A method for producing ds-cDNA using a nucleotide-immobilized support that includes a polynucleotide bound to an Insoluble Dish, said polynucleotide comprising at least one sequence complementary to a polyadenylic acid tail of mRNA, said method comprising the steps of:

(a) adding a sample solution containing mRNA to said nucleotide-immobilized support;

(b) incubating said sample solution and support, thereby creating a first liquid phase and a solid phase;

(c) removing the first liquid phase;

(d) adding a first reaction mixture to said solid phase, said solid phase comprising reverse transcriptase, dATP, DCTP, dGTP, and dTTP;

(e) incubating said first reaction mixture and solid phase;

(f) adding a second reaction mixture to said solid phase, said second reaction mixture comprising RNase, DNA polymerase, DNA ligase, dATP, dCTP, dGTP, dTTP; and

(g) incubating said second reaction mixture and solid phase.

20. A method according to Claim 19, wherein at least some of the dATP, DCTP, dGTP, or dTTP added in step (d) is labeled.

21. A method for producing sense ss-cDNA, comprising:

producing ds-cDNA according to the method of Claim 19;

heating said second reaction mixture and solid phase, thereby creating a second liquid phase containing said sense ss-cDNA; and

obtaining the second liquid phase.

22. A method for producing antisense ss-cDNA, comprising:

producing ds-cDNA according to Claim 19;

heating said second reaction mixture and solid phase, thereby creating a second liquid phase containing said sense ss-cDNA; and

obtaining the solid phase.

23. A method for producing sense ss-cDNA, comprising the steps of:

(a)   producing a ds-cDNA-immobilized support according to the method of Claim 1, wherein the sense cDNA and antisense cDNA form a ds-cDNA duplex in a liquid phase:

(b)   denaturing said ds-cDNA: and

(c)   obtaining said liquid phase.

24.   A method for producing sense ss-cDNA, comprising the steps of:

(a)   producing a ds-cDNA-immobilized support in a liquid phase, wherein one strand of said ds-cDNA is bound to an insoluble support, said one strand of said ds-cDNA comprising at least one sequence complementary to a polyadenylic acid tail of mRNA;

(b)   denaturing said ds-cDNA-immobilized support;

(c)   obtaining the liquid phase.

25.   A method for producing antisense ss-cDNA, comprising the steps of:

(a)   producing a ds-cDNA-immobilized support according to the method of Claim 1, wherein the sense cDNA and antisense cDNA form a ds-cDNA duplex in a liquid phase:

(b)   denaturing said ds-cDNA: and

(c)   removing said liquid phase, resulting in a solid phase containing said antisense ss-cDNA.

26.   The method of Claim 25, wherein said ds-cDNA comprises a restriction enzyme recognition site, additionally comprising digesting said antisense ds-cDNA with a restriction enzyme which recognizes said restriction enzyme recognition site.

27.   A method of placing a cDNA sequence into a vector in a particular orientation, comprising:

(a)   producing a ds-cDNA-immobilized support according to the method of Claim 1, wherein said polynucleotide bound to said insoluble support additionally comprises a first restriction enzyme recognition site:

(b)   adding a double stranded adaptor to said ds-cDNA, said adaptor comprising a second restriction enzyme recognition site different from the restriction enzyme recognition site on said polynucleotide bound to said insoluble support;

(c)   digesting said ds-cDNA with a restriction enzyme which recognizes said first restriction enzyme recognition site, and with a restriction enzyme which

recognizes said second restriction enzyme recognition site, thereby resulting in a non-bound ds-cDNA having a first sticky end at a first end thereof;

(d)   ligating said non-bound ds-cDNA to a vector having sequence complementary to said first sticky end at a first end thereof.

28.   The method of Claim 27, wherein the non-bound ds-cDNA of step (c) additionally results in a sticky end at a second end thereof and wherein the vector of step (d) additionally comprises a sequence complementary to said second sticky end at a second sticky end thereof.

29.   A method of immobilizing a first single stranded polynucleotide having at least one purine base to an insoluble support having an exposed sulfhydryl residue, comprising:

hybridizing said first single stranded polynucleotide with a second single stranded polynucleotide complementary to said first polynucleotide to form a double stranded complex, thereby protecting said purine base from further reaction;

reacting the 5' end of said first polynucleotide with a maleimide compound to produce a polynucleotide having a maleimide group at its 5' end; and

reacting the maleimide group at the 5' end of the first polynucleotide with said sulfhydrl residue.

30.   The method of Claim 29, additionally comprising denaturing said double stranded complex.

31.   The method of Claim 29, wherein said maleimide compound comprises sulfo-SMCC,

32.   The method of Claim 29, wherein said sulfhydryl residue is produced from an exposed amine residue on said insoluble support according to a method comprising:

reacting said amine residue with SATA to form a reacted complex;

deacetylating said reacted complex with hydroxylamine.

33.   The method of Claim 29, additionally comprising treating said support with a primary amine compound.

34.   A nucleotide-immobilized support, comprising:

(a)   an Insoluble Dish; and

(b)   a polynucleotide bound to said Insoluble Dish, said polynucleotide comprising at least one sequence complementary to a polyadenylic acid tail of

mRNA, at least one RNA promoter and at least one restriction enzyme recognition site.

35.   The nucleotide-immobilized support of Claim 34, wherein said polynucleotide comprises between 30 and 100 nucleotides.

36.   The nucleotide-immobilized support of Claim 34, wherein the Insoluble Dish comprises a microtiter plate.

37.   The nucleotide immobilized support of Claim 36, wherein the microtiter plate comprises at least two wells, and wherein each of the wells has a different polynucleotide immobilized therein.

38.   The nucleotide-immobilized support of Claim 34, wherein said polynucleotide contains a recognition site for a restriction enzyme selected from the group consisting of EcoRI NotI, Sma I and Sal I.

39.   The nucleotide-immobilized support of Claim 34, wherein said polynucleotide comprises a T7 RNA promoter or a SP6 RNA promoter.

40.   The nucleotide-immobilized support of Claim 34, wherein said polynucleotide comprises poly (dT) as the sequence complementary to a polyadenylic acid tail of mRNA.

41.   The nucleotide-immobilized support of Claim 34, wherein said polynucleotide contains a sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:13 and SEQ ID NO:14.

42.   The nucleotide-immobilized support of Claim 34, wherein said polynucleotide comprises at least two RNA promoters.

43.   The nucleotide-immobilized support of Claim 42, wherein said polynucleotide comprises at least two different RNA promoters.
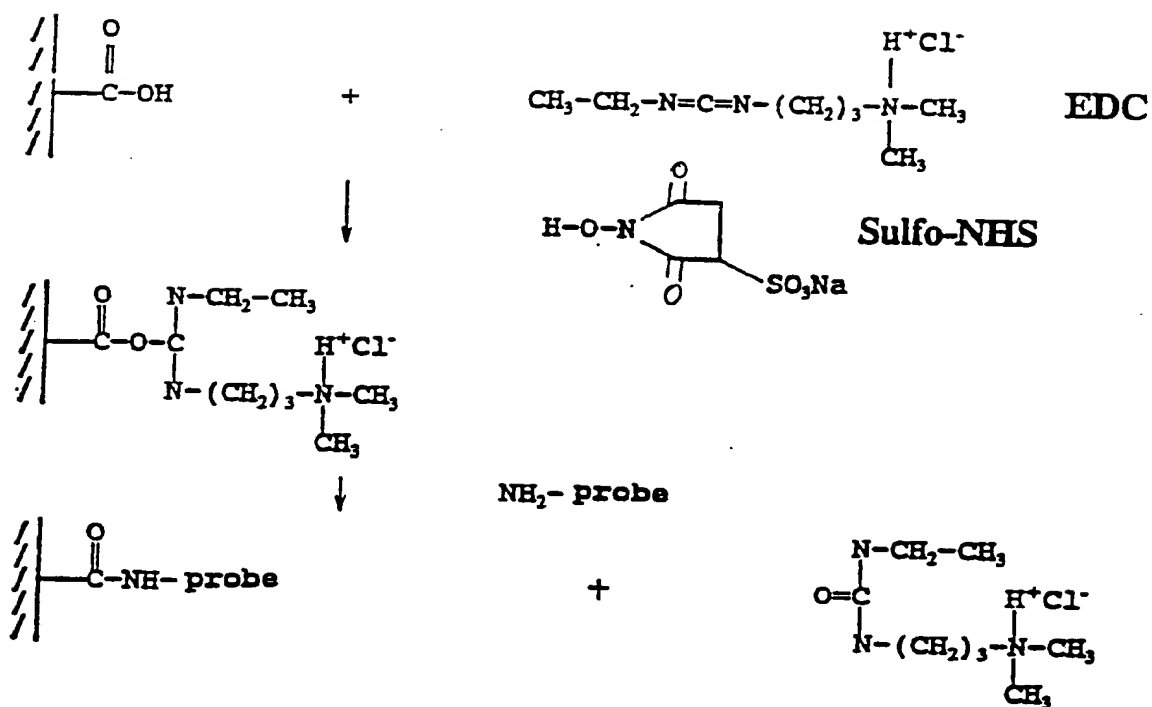
44.   The nucleotide-immobilized support of Claim 43, wherein said polynucleotide comprises a T7 RNA promoter and a SP6 RNA promoter.
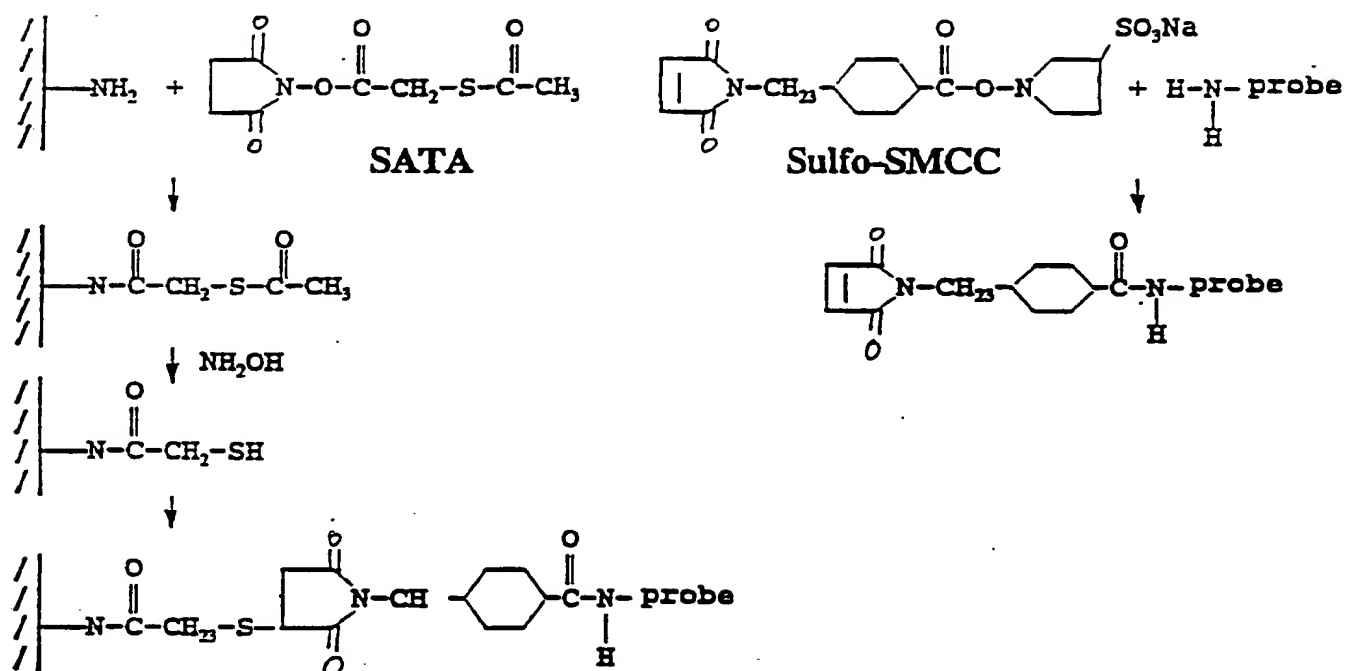
SATA:Succinimidyl-S-acetylthioacetate
Sulfo-SMCC:Sulfosuccinimidyl-4-(N-maleimidomethyl)
cyclohexane-1-carboxylate

Fig. 1

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EDC:1-ethyl-3-(3-dimethylaminopropyl)carbodiimode hydrochloride
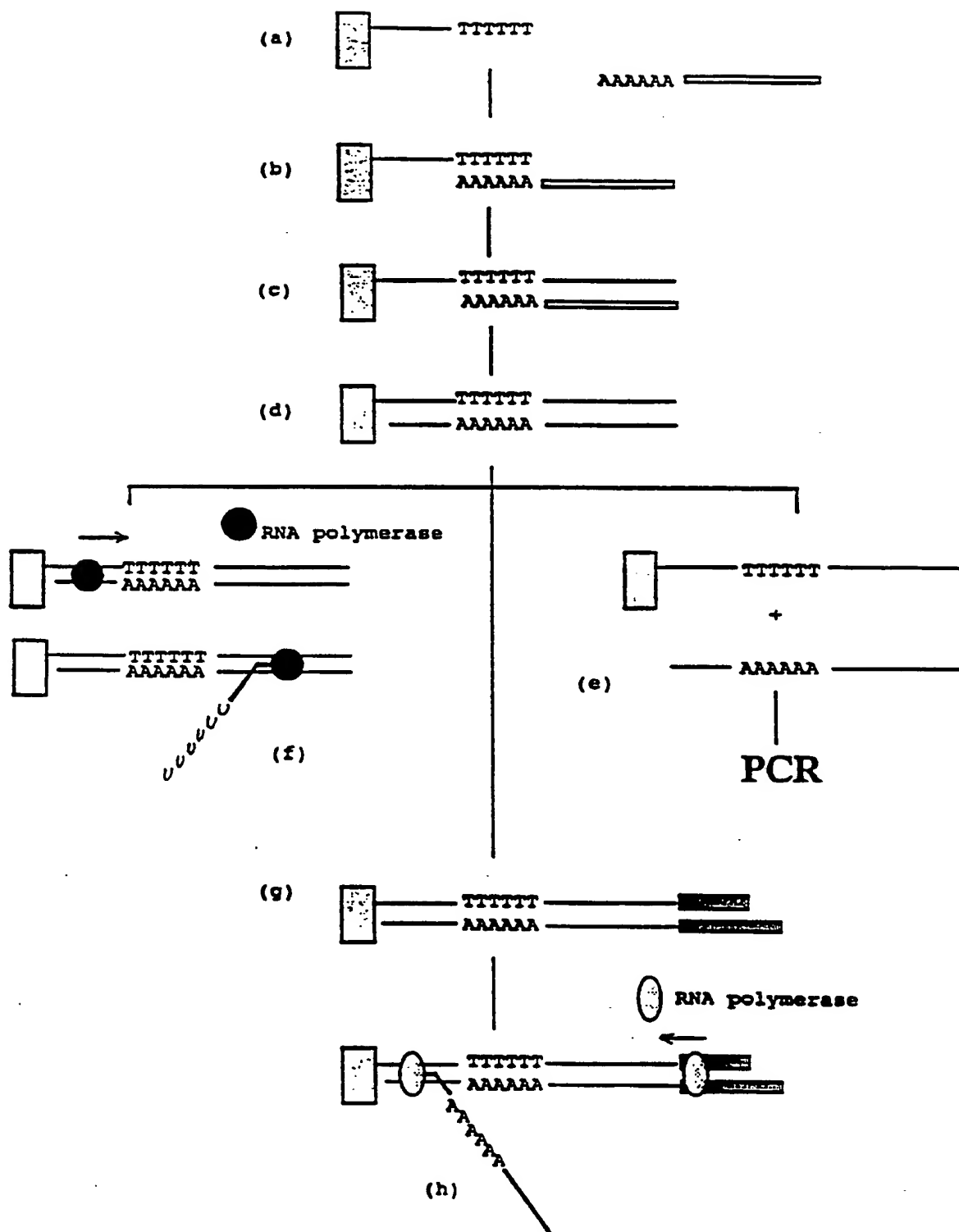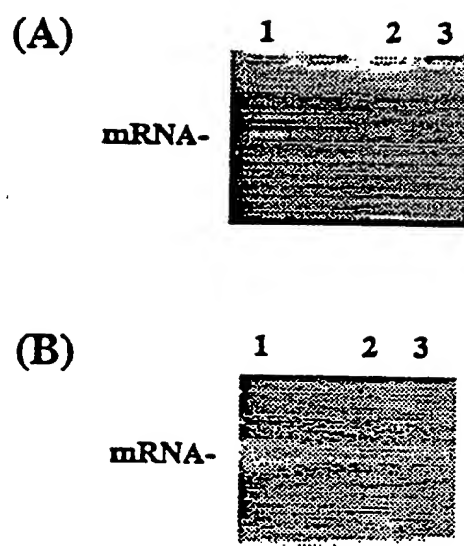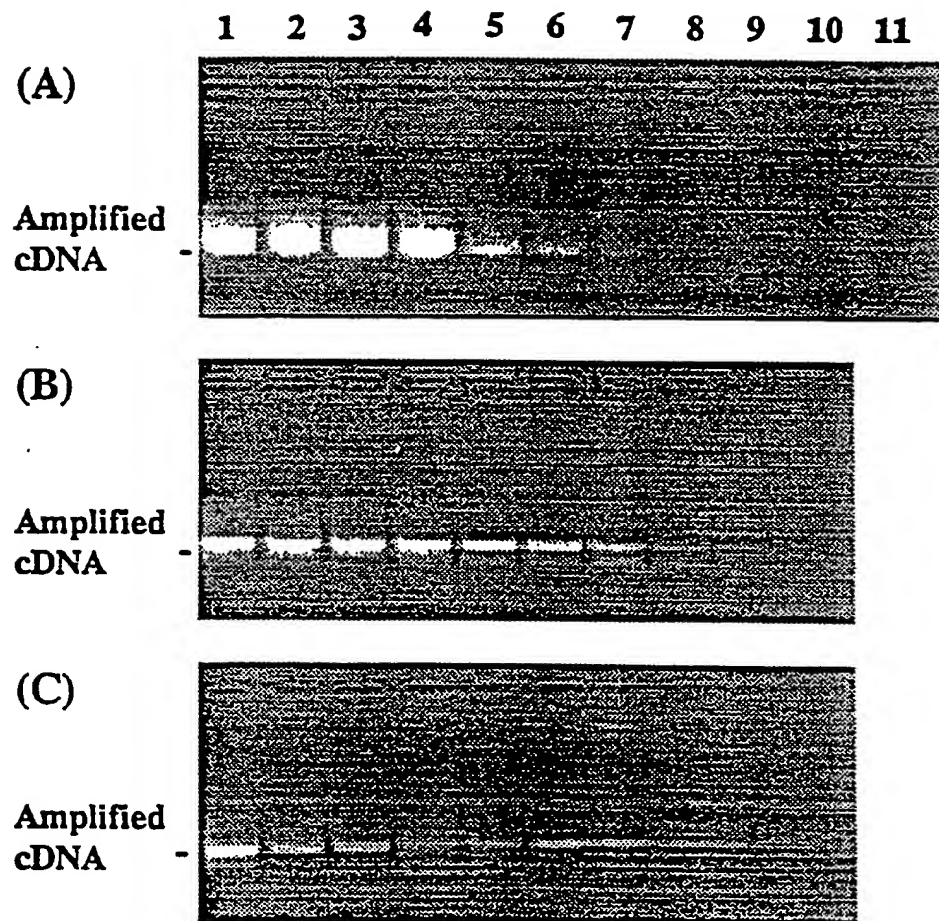Sulfo-NHS:N-hydroxysulfosuccinimide

Fig. 2

(a)

(b)

(c)

(d)

RNA polymerase

(e)

(f)

PCR

(g)

RNA polymerase

(h)

Fig. 3

(A)

1 2 3

mRNA-

(B)

1 2 3

mRNA-

Fig. 4

Fig. 5

Fig. 6

(A)   1   2   3

Amplified cDNA :

(B)   1   2   3

Amplified cDNA :

Fig. 7

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WO 93/15228

PCT/US93/01040

Amplified Gs protein DNA

Primers

Figure 8

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INTERNATIONAL SEARCH REPORT
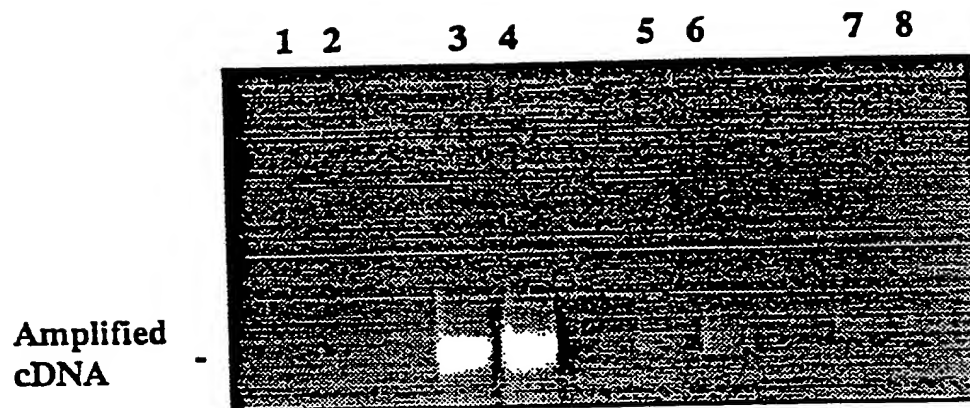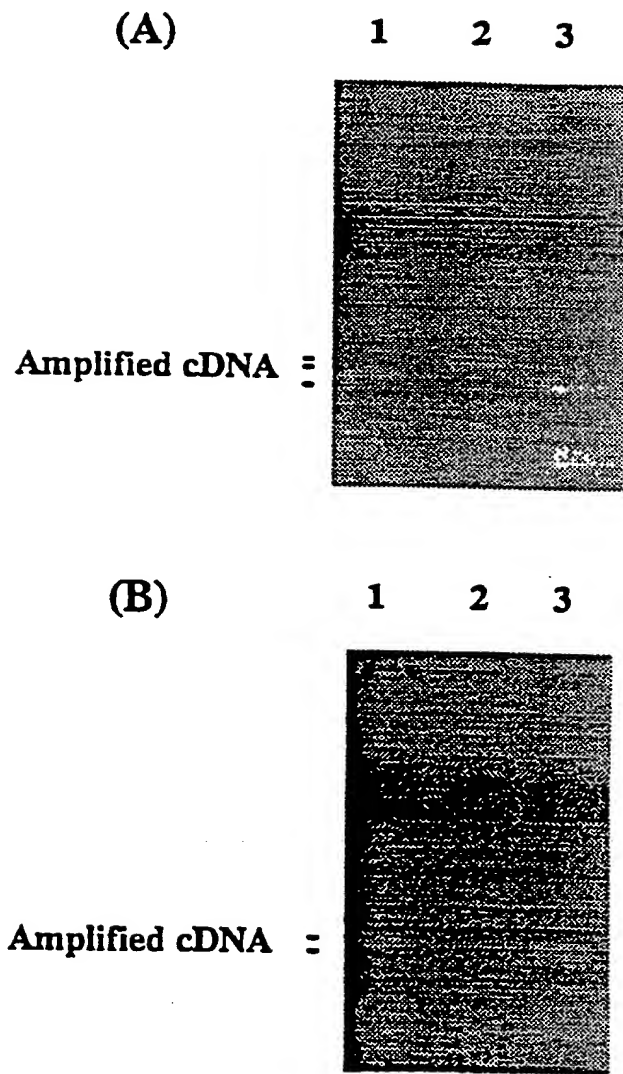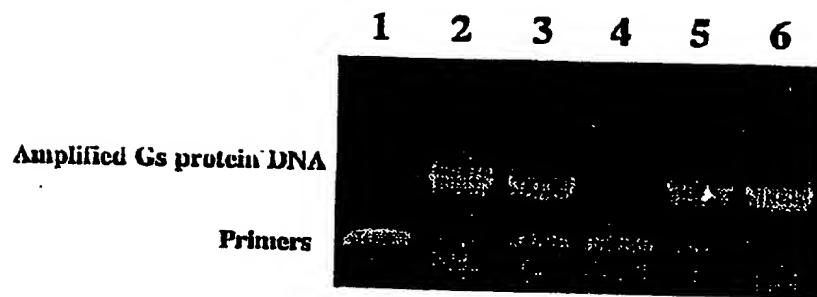
A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 01/68; C12P 19/34; C12N 09/10, 09/12; C07H 17/00, 15/12
US CL :435/6, 91, 193, 194; 536/23.1, 27.1, 28.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91, 193, 194; 536/23.1, 27.1, 28.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Medline

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/06042 (Hornes et al.) 14 June 1990, see entire document.	1-44
Y	EP, A, 0,329,822 (Davey et al.) 30 August 1989, see pages 7 and 15, claim 13.	1-28, 34-44
Y	Nucleic Acids Research, Volume 19, No. 14, issued 1991, I. Raineri et al., "Improved Efficiency for Single-Sided PCR by Creating a Reusable Pool of First-Strand cDNA coupled to a Solid Phase", page 4010, see Figure A.	1-28, 34-44

X	Further documents are listed in the continuation of Box C.		See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 APRIL 1993	14 MAY 1993

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	KATHLEEN L. CHOI
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Clinical Microbiology, Volume 28, No. 6, issued June 1990, S. Inouye et al., "Microplate Hybridization of Amplified Viral DNA Segment", pages 1469-1472, see entire document.	1-28, 34-44
Y	T.R. Sambrook et al., "Molecular Cloning, a Laboratory Manual", published 1989 by Cold Spring Harbor Laboratory Press (N.Y.), see pages 7.26-7.29 and 8.21-8.35.	1-28, 34-44
Y	Gene, Volume 52, issued 1987, M.J. Palazzolo et al., "A Family of Lambda Phage cDNA Cloning Vectors, λSWAJ, Allowing the Amplification of RNA Sequences", pages 197-206, see Figure 2.	27-28, 42-44
Y	EP, A, 0,152,886 (Dattagupta) 28 August 1985, see Figure 2.	29-33
Y	Analytical Biochemitry, Volume 164, No. 2, issued 01 August 1987, R. Bischoff et al., "Introduction of 5'-Terminal Functional Groups into Synthetic Oligonucleotides for Selective Immobilization", pages 336-344, see entire document.	29-33
Y	Nucleic Acids Research, Volume 19, No. 6, issued 25 March 1991, S. Stamm et al., "Sanchored PCR: PCR with cDNA Coupled to a Solid Phase", page 1350, entire document.	29-33
Y	Analytical Biochemistry, Volume 132, issued 1983, R.J.S. Duncan et al., "A New Reagent Which May be Used to Introduce Sulfhydryl Groups into Proteins, and its Use in the Preparation of Conjugates for Immunoassay", pages 68-74, see entire document.	32